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Invention: POLYNUCLEOTIDE AND ITS USE FOR MODULATING A DEFENCE RESPONSE IN PLANTS

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SPECIFICATION

POLYNUCLEOTIDE AND ITS USE FOR MODULATING A DEFENCE RESPONSE IN PLANTS

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The present invention relates to stimulating a defence response in plants, with a view to providing the plants with enhanced pathogen resistance. More specifically, it has resulted from cloning of the barley *Mlo* gene, various mutant *mlo* alleles, and a number of homologues from various species. The *Mlo* gene has been isolated using a positional cloning approach which has never previously been successful in Barley. Details and discussion are provided below. Wild-type *Mlo* exerts a negative regulatory function on a pathogen defence response, such that mutants exhibit a defence response in the absence of pathogen. In accordance with the present invention, down-regulation or out-competition of *Mlo* function may be used to stimulate a defence response in transgenic plants, conferring increased pathogen resistance.

Mutations have been described in several plants in which defence responses to pathogens appear to be constitutively expressed. Mutation-induced recessive alleles (*mlo*) of the barley *Mlo* locus exhibit a leaf lesion phenotype and confer an apparently durable, broad spectrum resistance to the powdery mildew pathogen, *Erysiphe graminis* f sp *hordei*.

Resistance responses to the powdery mildew pathogen have been genetically well characterized (Wiberg, 1974; Søgaard and Jørgensen, 1988; Jørgensen, 1994). In most analyzed cases resistance is specified by race-specific resistance genes following the rules of Flor's gene-for-gene hypothesis (Flor, 1971). In this type of plant/pathogen interaction, resistance

is specified by and dependent on the presence of two complementary genes, one from the host and one from the fungal pathogen. The complementary genes have been termed operationally (pathogen) resistance ("*R*") gene and avirulence gene, respectively. Most of the powdery mildew resistance genes (*Mlx*) act as dominant or semidominant traits (Jørgensen, 1994).

Monogenic resistance mediated by recessive (*mlo*) alleles of the *Mlo* locus is different. Apart from being recessive, it differs from race-specific resistance to single pathogen strains in that (i) it confers broad spectrum resistance to almost all known isolates of the pathogen (ii) *mlo* resistance alleles have been obtained by mutagen treatment of any tested susceptible wild type (*Mlo*) variety, and (iii) *mlo* resistance alleles exhibit a defence mimic phenotype in the absence of the pathogen (Wolter *et al.* , 1993). Thus, the genetic data indicate the *Mlo* wild type allele exerts a negative regulatory function on defence responses to pathogen attack.

Resistance mediated by *mlo* alleles is currently widely used in barley breeding and an estimated 10 million hectares are annually planted in Europe with seeds of this genotype. A '*mlo* like' inherited resistance to powdery mildew in other cereal plants has not been reported so far although the fungus is a relevant pathogen in wheat (attacked by *Erysiphe graminis* f sp *tritici*), oat (attacked by *E. g.* f sp *avenae*), and rye (attacked by *E. g.* f sp *secalis*). Because cereals are morphologically, genetically and biochemically highly related to each other (Moore et al., 1995), one would predict the

existence of homologous genes in these species. The failure to have found a '*mlo* like' inherited resistance in wheat and oat is probably due to their hexaploid genomes, making it difficult to obtain by mutagenesis defective alleles in all six gene copies, and the chance of all such mutations occurring in Nature is remote. The failure to have found a *mlo* equivalent in other cereals is probably due to insignificant amount of mutational analysis in these species and complications as a result of their outbreeding nature (e.g. rye).

RFLP markers closely linked to *Mlo* on barley chromosome 4 were previously identified on the basis of a *mlo* backcross line collection containing *mlo* alleles from six genetic backgrounds (Hinze *et al.* , 1991). The map position of *Mlo* on the basis of RFLP markers was consistent with its chromosomal localization as determined by a previous mapping with morphological markers (Jørgensen, 1977).

Having identified an ~3cM genetic interval containing *Mlo* bordered by genetic markers, we decided to attempt to isolate the gene via positional cloning.

However, there is no documented example of a successful positional cloning attempt of a barley gene. We were faced with a number of difficulties.

Firstly, the genome of barley ($5.3x10^9$ bp/haploid genome equivalent; Bennett and Smith, 1991) has almost double the size of the human genome and because the total genetic map covers ~1.800 cM (Becker *et al.*, 1995) we were confronted with a very unfavourable ratio of genetic and physical distances (1 cM

corresponds to ~ 3 Mb).

Secondly, a high resolution genetic map had to be constructed around *Mlo* enabling the positioning of linked markers with a precision of better than 0.1 cM.

Thirdly, we aimed to physically delimit the target gene and both flanking DNA markers on individual large insert genomic clones, a procedure later termed "chromosome landing" (Tanksley et al., 1995). For this purpose, a complete barley YAC library from barley Megabase DNA had to be constructed with an average insert size of 500-600 kb, which was unprecedented.

Fourthly, we had to prepare unusual genetic tools that enabled us to identify the *Mlo* gene within a physically delimited region without the need for a time consuming generation of barley transgenic plants and testing of different candidate genes. We used for our studies ten characterized radiation- or chemically-induced *mlo* mutants (Jørgensen, 1992). For a conclusive chain of evidence of the gene isolation we decided to depend upon a functional restoration of the wild type *Mlo* allele starting out from characterized *mlo* defective alleles. For this purpose, we performed *mlo* heteroallelic crosses and isolated susceptible intragenic *Mlo* recombinants. The sequence analysis of these proves the function of the described gene.

The cloning of the barley *Mlo* gene and homologues, including homologues from other plant species, gives rise to a number of practical applications, reflected in the various aspects of the present invention.

According to a first aspect of the present invention there is provided a nucleic acid molecule comprising a nucleotide sequence encoding a peptide with Mlo function. Those skilled in the art will appreciate that "Mlo function" refers to the ability to suppress a defence response, said defence response being race and/or pathogen independent and autonomous of the presence of a pathogen, such as, for example, the *Mlo* gene of barley, the *Acd* gene and the *Lsd* gene of *Arabidopsis*.

mlo mutations that down-regulate or disrupt functional expression of the wild-type Mlo sequence are recessive, such that they are complemented by expression of a wild-type sequence. Thus "*Mlo* function" can be determined by assessing the level of constitutive defence response and/or susceptibility of the plant to a pathogen such as, for example, powdery mildew or rust (e.g. yellow rust). Accordingly, a putative nucleotide sequence with *Mlo* function can be tested upon complementation of a suitable *mlo* mutant. The term "mlo function" is used to refer to sequences which confer a *mlo* mutant phenotype on a plant.

The capitalisation of "Mlo" and non-capitalisation of "mlo" is thus used to differentiate between "wild-type" and "mutant" function.

A *mlo* mutant phenotype is characterised by the exhibition of an increased resistance against one or more pathogens, which is race and/or pathogen independent and autonomous of the presence of a pathogen.

The test plant may be monocotyledonous or dicotyledonous.

Suitable monocots include any of barley, rice, wheat, maize or oat, particularly barley. Suitable dicots include *Arabidopsis*.

Nucleic acid according to the invention may encode a polypeptide comprising the amino acid sequence shown in Figure 2, or an allele, variant, derivative or mutant, or homologue, thereof.

Nucleic acid according to the present invention may have the sequence of a *Mlo* gene of barley, or be a mutant, variant (or derivative) or allele of the sequence provided, or a homologue thereof. Preferred mutants, variants and alleles are those which encode a sequence which retains a functional characteristic of the wild-type gene, especially the ability to suppress a defence response as discussed herein. Other preferred mutants, variants and alleles encode a sequence which, in a homozygote, cause constitutive activation of a defence response, or at least promotes activation of a defence response (i.e. is a *mlo* mutant sequence), e.g. by reducing or wholly or partly abolishing Mlo function. Preferred mutations giving *mlo* mutant sequences are shown in Table 1. Changes to a sequence, to produce a mutant, derivative or variant, may be by one or more of addition, insertion, deletion or substitution of one or more nucleotides in the nucleic acid, leading to the addition, insertion, deletion and/or substitution of one or more amino acids. Of course, changes to the nucleic acid which make no difference to the encoded amino acid sequence are included. Particular variants, mutants, alleles and

derivatives are discussed further below, as well as homologues.

A preferred nucleic acid sequence according to an aspect of the present invention is shown in Figure 2 along with the predicted amino acid sequence. Nucleic acid may be subject to alteration by way of substitution of nucleotides and/or a combination of addition, insertion and/or substitution of one or more nucleotides with or without altering the encoded amino acids sequence (by virtue of the degeneracy of the genetic code).

As discussed below, further aspects of the present invention provide homologues of the *Mlo* sequence shown in Figure 2, including from rice (genomic sequence Figure 5, bottom line, cDNA sequence Figure 10, amino acid sequence Figure 13) and barley (genomic sequence Figure 6, bottom line, cDNA sequence Figure 11, amino acid sequence Figure 14); also Table 5B (nucleotide sequences) and Figure 5A (amino acid sequences) show homologous EST's from rice and *Arabidopsis*.

The present invention also provides a vector which comprises nucleic acid with any one of the provided sequences, preferably a vector from which a product can be expressed. The vector is preferably suitable for transformation into a plant cell and/or a microbial cell. The invention further encompasses a host cell transformed with such a vector, especially a plant cell or a microbial cell (e.g. *Agrobacterium tumefaciens*). Thus, a host cell, such as a plant cell, comprising nucleic acid according to the present invention is provided. Within the cell, the nucleic acid may be incorporated within the nuclear

genome, i.e. a chromosome. There may be more than one heterologous nucleotide sequence per haploid genome.

A vector comprising nucleic acid according to the present invention need not include a promoter, particularly if the vector is to be used to introduce the nucleic acid into cells for recombination into the genome.

Nucleic acid molecules and vectors according to the present invention may be provided in a form isolated and/or purified from their natural environment, in substantially pure or homogeneous form, or free or substantially free of nucleic acid or genes of the species of interest or origin other than the relevant sequence. Nucleic acid according to the present invention may comprise cDNA, RNA, genomic DNA and may be wholly or partially synthetic. The term "isolate" may encompass all these possibilities.

The present invention also encompasses the expression product of any of the nucleic acid sequences disclosed and methods of making the expression product by expression from encoding nucleic acid therefore under suitable conditions in suitable host cells, e.g. *E. coli*. Those skilled in the art are well able to construct vectors and design protocols for expression and recovery of products of recombinant gene expression. Suitable vectors can be chosen or constructed, containing one or more appropriate regulatory sequences, including promoter sequences, terminator fragments, polyadenylation sequences, enhancer sequences, marker genes and other sequences as appropriate. For further details see, for

example, Molecular Cloning: a Laboratory Manual: 2nd edition, Sambrook et al, 1989, Cold Spring Harbor Laboratory Press. Transformation procedures depend on the host used, but are well known. Many known techniques and protocols for manipulation of nucleic acid, for example in preparation of nucleic acid constructs, mutagenesis, sequencing, introduction of DNA into cells and gene expression, and analysis of proteins, are described in detail in *Short Protocols in Molecular Biology*, Second Edition, Ausubel et al. eds., John Wiley & Sons, 1992. The disclosures of Sambrook et al. and Ausubel et al. are incorporated herein by reference, along with all other documents mentioned.

Purified Mlo protein, or a fragment, mutant or variant thereof, e.g. produced recombinantly by expression from encoding nucleic acid therefor, may be used to raise antibodies employing techniques which are standard in the art. Antibodies and polypeptides comprising antigen-binding fragments of antibodies may be used in identifying homologues from other species as discussed further below.

Methods of producing antibodies include immunising a mammal (eg human, mouse, rat, rabbit, horse, goat, sheep or monkey) with the protein or a fragment thereof. Antibodies may be obtained from immunised animals using any of a variety of techniques known in the art, and might be screened, preferably using binding of antibody to antigen of interest. For instance, Western blotting techniques or immunoprecipitation may be used (Armitage et al, 1992, Nature 357: 80-82).

Antibodies may be polyclonal or monoclonal.

As an alternative or supplement to immunising a mammal, antibodies with appropriate binding specificity may be obtained from a recombinantly produced library of expressed immunoglobulin variable domains, eg using lambda bacteriophage or filamentous bacteriophage which display functional immunoglobulin binding domains on their surfaces; for instance see WO92/01047.

Antibodies raised to a polypeptide or peptide can be used in the identification and/or isolation of homologous polypeptides, and then the encoding genes. Thus, the present invention provides a method of identifying or isolating a polypeptide with Mlo or mlo function (in accordance with embodiments disclosed herein), comprising screening candidate peptides or polypeptides with a polypeptide comprising the antigen-binding domain of an antibody (for example whole antibody or a fragment thereof) which is able to bind an Mlo or mlo peptide, polypeptide or fragment, variant or variant thereof or preferably has binding specificity for such a peptide or polypeptide, such as having an amino acid sequence identified herein. Specific binding members such as antibodies and polypeptides comprising antigen binding domains of antibodies that bind and are preferably specific for a Mlo or mlo peptide or polypeptide or mutant, variant or derivative thereof represent further aspects of the present invention, as do their use and methods which employ them.

Candidate peptides or polypeptides for screening may for

instance be the products of an expression library created using nucleic acid derived from an plant of interest, or may be the product of a purification process from a natural source.

A peptide or polypeptide found to bind the antibody may be isolated and then may be subject to amino acid sequencing. Any suitable technique may be used to sequence the peptide or polypeptide either wholly or partially (for instance a fragment of a polypeptide may be sequenced). Amino acid sequence information may be used in obtaining nucleic acid encoding the peptide or polypeptide, for instance by designing one or more oligonucleotides (e.g. a degenerate pool of oligonucleotides) for use as probes or primers in hybridisation to candidate nucleic acid, or by searching computer sequence databases, as discussed further below.

A further aspect of the present invention provides a method of identifying and cloning *Mlo* homologues from plants, including species other than Barley, which method employs a nucleotide sequence derived from that shown in Figure 2. Further similar aspects employ a nucleotide sequence derived from any of the other Figures provided herein. Nucleic acid libraries may be screened using techniques well known to those skilled in the art and homologous sequences thereby identified then tested. The provision of sequence information for the *Mlo* gene of Barley and various homologues enables the obtention of homologous sequences from Barley and other plant species, as exemplified further herein.

Also, one can easily derive PCR primers based on putative

within the sequence shown in Figure 2, a single amino acid change with respect to the sequence shown in Figure 2, or 2, 3, 4, 5, 6, 7, 8, or 9 changes, about 10, 15, 20, 30, 40 or 50 changes, or greater than about 50, 60, 70, 80 or 90 changes. In addition to one or more changes within the amino acid sequence shown in Figure 2, a mutant, allele, variant or derivative amino acid sequence may include additional amino acids at the C-terminus and/or N-terminus.

As is well-understood, homology at the amino acid level is generally in terms of amino acid similarity or identity. Similarity allows for "conservative variation", i.e. substitution of one hydrophobic residue such as isoleucine, valine, leucine or methionine for another, or the substitution of one polar residue for another, such as arginine for lysine, glutamic for aspartic acid, or glutamine for asparagine. Similarity may be as defined and determined by the TBLASTN program, of Altschul *et al*. (1990) *J. Mol. Biol*. 215: 403-10, which is in standard use in the art, or, and this may be preferred, the standard program BestFit, which is part of the Wisconsin Package, Version 8, September 1994, (Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA, Wisconsin 53711). BestFit makes an optimal alignment of the best segment of similarity between two sequences. Optimal alignments are found by inserting gaps to maximize the number of matches using the local homology algorithm of Smith and Waterman

Homology may be over the full-length of the relevant

exon sequences, which might be identified by comparison with the *Mlo* sequence provided in Figure 2 wherein exons are highlighted, and perform RT-PCR with total RNA from the plant of interest, e.g. barley and rice for the homologues shown in Figures 5 and 6, with cDNA and amino acid sequences shown in other figures herein.

The homologues whose nucleotide sequences are given and whose amino acid sequences are given or are deducible represent and provide further aspects of the present invention in accordance with those disclosed for the Barley gene shown in Figure 2.

The present invention also extends to nucleic acid encoding a *Mlo* homologue obtained using a nucleotide sequence derived from that shown in Figure 2, or the amino acid sequence shown in Figure 2. Preferably, the nucleotide sequence and/or amino acid sequence shares homology with the sequence encoded by the nucleotide sequence of Figure 2, preferably at least about 50%, or at least about 55%, or at least about 60%, or at least about 65%, or at least about 70%, or at least about 75%, or at least about 80% homology, or at least about 85% homology, or at least about 90% homology, most preferably at least about 95% homology. "Homology" in relation to an amino acid sequence may be used to refer to identity or similarity, preferably identity. High levels of amino acid identity may be limited to functionally significant domains or regions.

A mutant, allele, variant or derivative amino acid sequence in accordance with the present invention may include

sequence shown herein, or may more preferably be over a contiguous sequence of about or greater than about 20, 25, 30, 33, 40, 50, 67, 133, 167, 200, 233, 267, 300, 333, 400, 450, 500, 550, 600 or more amino acids or codons, compared with the relevant amino acid sequence or nucleotide sequence as the case may be.

The EST sequences provided herein, have on average 70% similarity and 50% identity with the Mlo amino acid sequence of Figure 2. We show that the rice homologue (Figure 5) and barley homologue (Figure 6) have an amino acid identity of 81% (amino acid sequences shown in Figure 13 and Figure 14).

In certain embodiments, an allele, variant, derivative, mutant or homologue of the specific sequence may show little overall homology, say about 20%, or about 25%, or about 30%, or about 35%, or about 40% or about 45%, with the specific sequence. However, in functionally significant domains or regions the amino acid homology may be much higher. Putative functionally significant domains or regions can be identified using processes of bioinformatics, including comparison of the sequences of homologues. Functionally significant domains or regions of different polypeptides may be combined for expression from encoding nucleic acid as a fusion protein. For example, particularly advantageous or desirable properties of different homologues may be combined in a hybrid protein, such that the resultant expression product, with Mlo or mlo function, may comprise fragments of various parent proteins.

The nucleotide sequence information provided herein, or

any part thereof, may be used in a data-base search to find homologous sequences, expression products of which can be tested for *Mlo* or *mlo* function. These may have ability to complement a *mlo* mutant phenotype in a plant or may, upon expression in a plant, confer a *mlo* phenotype.

In public sequence databases we recently identified several homologues for the sequence of Figure 2. We have already found homologues in rice and barley, and the dicot. *Arabidopsis*.

By sequencing homologues, studying their expression patterns and examining the effect of altering their expression, genes carrying out a similar function to *Mlo in* Barley are obtainable. Of course, mutants, variants and alleles of these sequences are included within the scope of the present invention in the same terms as discussed above for the Barley gene.

Homology between the homologues as disclosed herein, may be exploited in the identification of further homologues, for example using oligonucleotides (e.g. a degenerate pool) designed on the basis of sequence conservation.

According to a further aspect, the present invention provides a method of identifying or a method of cloning a *Mlo* homologue, e.g. from a species other than Barley, the method employing a nucleotide sequence derived from that shown in Figure 2 or that shown in any of the other Figures herein. For instance, such a method may employ an oligonucleotide or oligonucleotides which comprises or comprise a sequence or

sequences that are conserved between the sequences of Figures 2 and/or 5 and/or 6 and/or 10 and/or 11 and/or 12, or encoding an amino acid sequence conserved between Figure 2 and/or 7 and/or 13 and/or 14 and/or 15 to search for homologues. Thus, a method of obtaining nucleic acid is provided, comprising hybridisation of an oligonucleotide or a nucleic acid molecule comprising such an oligonucleotide to target/candidate nucleic acid. Target or candidate nucleic acid may, for example, comprise a genomic or cDNA library obtainable from an organism known to contain or suspected of containing such nucleic acid, either monocotyledonous or dicotyledonous. Successful hybridisation may be identified and target/candidate nucleic acid isolated for further investigation and/or use.

Hybridisation may involve probing nucleic acid and identifying positive hybridisation under suitably stringent conditions (in accordance with known techniques) and/or use of oligonucleotides as primers in a method of nucleic acid amplification, such as PCR. For probing, preferred conditions are those which are stringent enough for there to be a simple pattern with a small number of hybridisations identified as positive which can be investigated further. It is well known in the art to increase stringency of hybridisation gradually until only a few positive clones remain.

As an alternative to probing, though still employing nucleic acid hybridisation, oligonucleotides designed to amplify DNA sequences may be used in PCR reactions or other methods involving amplification of nucleic acid, using routine

procedures. See for instance "PCR protocols; A Guide to Methods and Applications", Eds. Innis *et al*, 1990, Academic Press, New York.

Preferred amino acid sequences suitable for use in the design of probes or PCR primers for some purposes are sequences conserved (completely, substantially or partly) between at least two Mlo peptides or polypeptides encoded by genes able to suppress a defence response in a plant, e.g. with any of the amino acid sequences of any of the various figures herein and/or encoded by the nucleotide sequences of any of the various figures herein.

On the basis of amino acid sequence information oligonucleotide probes or primers may be designed, taking into account the degeneracy of the genetic code, and, where appropriate, codon usage of the organism from the candidate nucleic acid is derived.

Preferably an oligonucleotide in accordance with certain embodiments of the invention, e.g. for use in nucleic acid amplification, is up to about 50 nucleotides, or about 40 nucleotides or about 30 or fewer nucleotides in length (e.g. 18, 21 or 24).

Assessment of whether or not such a PCR product corresponds to *Mlo* homologue genes may be conducted in various ways. A PCR band from such a reaction might contain a complex mix of products. Individual products may be cloned and each one individually screened. It may be analysed by transformation to assess function on introduction into a plant

of interest.

As noted, nucleic acid according to the present invention is obtainable using oligonucleotides, designed on the basis of sequence information provided herein, as probes or primers. Nucleic acid isolated and/or purified from one or more cells of barley or another plant (see above), or a nucleic acid library derived from nucleic acid isolated and/or purified from the plant (e.g. a cDNA library derived from mRNA isolated from the plant), may be probed under conditions for selective hybridisation and/or subjected to a specific nucleic acid amplification reaction such as the polymerase chain reaction (PCR). The nucleic acid probed or used as template in the amplification reaction may be genomic DNA, cDNA or RNA. If necessary, one or more gene fragments may be ligated to generate a full-length coding sequence.

We have tested several PCR primers derived from the *Mlo* sequence disclosed herein to test their specificity for amplifying nucleic acid according to the present invention, using both barley genomic DNA and RT-PCR templates. The latter was synthesized from barley polyA$^+$ RNA. In each case we were able to amplify the expected *Mlo* derived gene fragments as shown by cloning and subsequent DNA sequencing of the PCR products. Full length cDNA clones can be obtained as described by 5′ and 3′ RACE technology if RT-PCR products are used as templates.

Examples of primers tested include:

	25L	5'-GTG CAT CTG CGT GTG CGT A-3'
	25LN	5'-GTG TGC GTA CCT GGT AGA G-3'
	25R	5'-AAC GAC GTC TGG TGC GTG-3'
	33	5'-TGC AGC TAT ATG ACC TTC CCC CTC-3'
	37	5'-GGA CAT GCT GAT GGC TCA GA-3'
	38	5'-CAG AAC TTG TCT CAT CCC TG-3
	38A	5'-GGC TAT ACA TTG GGA CTA ACA-3'
	38B	5'-CGA ATC ATC ACA TCC TAT GTT-3'
	39	5'-GCA AGT TCG ACT TCC AC-3'
	39A	5'-TCG ACT TCC ACA AGT ACA TCA-3'
	53	5'-AGC GTA CCT GCG TAC GTA G-3'

Various primer combinations have been tested: 38/39A; 38/39; 38/33; 38/37; 38A/39A; 38B/39A; 38/25L; 38/25LN; 25R/25L; 25R/25LN; 25R/53.

Various aspects of the present invention include the obtainable nucleic acid, methods of screening material, e.g. cell lysate, nucleic acid preparations, for the presence of nucleic acid of interest, methods of obtaining the nucleic acid, and the primers and primer combinations given above.

The sequence information provided herein also allows the design of diagnostic tests for determination of the presence of a specific mlo resistance allele, or a susceptibility allele (e.g. wild-type), in any given plant, cultivar, variety, population, landrace, part of a family or other selection in a breeding programme or other such genotype. A diagnostic test

may be based on determination of the presence or absence of a particular allele by means of nucleic acid or polypeptide determination.

At the nucleic acid level, this may involve hybridisation of a suitable oligo- or poly-nucleotide, such as a fragment of the Mlo gene or a homologue thereof, including any homologue disclosed herein, or any particular allele, such as an allele which gives an *mlo* phenotype, such as any such allele disclosed herein. The hybridisation may involve PCR designed to amplify a product from a given allelic version of *mlo*, with subsequent detection of an amplified product by any of a number of possible methods including but not limited to gel electrophoresis, capillary electrophoresis, direct hybridisation of nucleotide sequence probes and so on. A diagnostic test may be based on PCR designed to amplify various alleles or any allele from the Mlo locus, with a test to distinguish the different possible alleles by any of a number of possible methods, including DNA fragment size, restriction site variation (e.g. CAPS - cleaved amplified polymorphic sites) and so on. A diagnostic test may also be based on a great number of possible variants of nucleic acid analysis that will be apparent to those skilled in the art, such as use of a synthetic *mlo*-derived sequence as a hybridisation probe.

Broadly, the methods divide into those screening for the presence of nucleic acid sequences and those that rely on detecting the presence or absence of a polypeptide. The methods may make use of biological samples from one or more

plants or cells that are suspected to contain the nucleic acid sequences or polypeptide.

Exemplary approaches for detecting nucleic acid or polypeptides include analysing a sample from the plant or plant cell by:

(a) comparing the sequence of nucleic acid in the sample with all or part of the nucleotide sequence shown in Figure 7 to determine whether the sample from the patient contains a mutation;

(b) determining the presence in the sample of a polypeptide including the amino acid sequence shown in Figure 2 or a fragment thereof and, if present, determining whether the polypeptide is full length, and/or is mutated, and/or is expressed at the normal level;

(c) performing DNA fingerprinting to compare the restriction pattern produced when a restriction enzyme cuts nucleic acid in the sample with the restriction pattern obtained from the nucleotide sequence shown in Figure 7 or from a known mutant, allele or variant thereof;

(d) contacting the sample with a specific binding member capable of binding to nucleic acid including the nucleotide sequence as set out in Figure 7 or a fragment thereof, or a mutant, allele or variant thereof, the specific binding member including nucleic acid hybridisable with the sequence of Figure 7 or a polypeptide including a binding domain with specificity for nucleic acid including the sequence of Figure 7 or the polypeptide encoded by it, or a mutated form thereof, and

determining binding of the specific binding member;

(e) performing PCR involving one or more primers based on the nucleotide sequence shown in Figure 7 to screen the sample for nucleic acid including the nucleotide sequence of Figure 7 or a mutant, allele or variant thereof.

When screening for a resistance allele nucleic acid, the nucleic acid in the sample will initially be amplified, e.g. using PCR, to increase the amount of the analyte as compared to other sequences present in the sample. This allows the target sequences to be detected with a high degree of sensitivity if they are present in the sample. This initial step may be avoided by using highly sensitive array techniques that are becoming increasingly important in the art.

A variant form of the gene may contain one or more insertions, deletions, substitutions and/or additions of one or more nucleotides compared with the wild-type sequence (such as shown in Table 1) which may or may not disrupt the gene function. Differences at the nucleic acid level are not necessarily reflected by a difference in the amino acid sequence of the encoded polypeptide. However, a mutation or other difference in a gene may result in a frame-shift or stop codon, which could seriously affect the nature of the polypeptide produced (if any), or a point mutation or gross mutational change to the encoded polypeptide, including insertion, deletion, substitution and/or addition of one or more amino acids or regions in the polypeptide. A mutation in a promoter sequence or other regulatory region may prevent or

reduce expression from the gene or affect the processing or stability of the mRNA transcript.

Tests may be carried out on preparations containing genomic DNA, cDNA and/or mRNA. Testing cDNA or mRNA has the advantage of the complexity of the nucleic acid being reduced by the absence of intron sequences, but the possible disadvantage of extra time and effort being required in making the preparations. RNA is more difficult to manipulate than DNA because of the wide-spread occurrence of RN'ases.

Nucleic acid in a test sample may be sequenced and the sequence compared with the sequence shown in Figure 2, or other figure herein, to determine whether or not a difference is present. If so, the difference can be compared with known susceptibility alleles (e.g. as summarised in Table 1) to determine whether the test nucleic acid contains one or more of the variations indicated, or the difference can be investigated for association with disease resistance.

The amplified nucleic acid may then be sequenced as above, and/or tested in any other way to determine the presence or absence of a particular feature. Nucleic acid for testing may be prepared from nucleic acid removed from cells or in a library using a variety of other techniques such as restriction enzyme digest and electrophoresis.

Nucleic acid may be screened using a variant- or allele-specific probe. Such a probe corresponds in sequence to a region of the gene, or its complement, containing a sequence alteration known to be associated with disease resistance.

Under suitably stringent conditions, specific hybridisation of such a probe to test nucleic acid is indicative of the presence of the sequence alteration in the test nucleic acid. For efficient screening purposes, more than one probe may be used on the same test sample.

Allele- or variant-specific oligonucleotides may similarly be used in PCR to specifically amplify particular sequences if present in a test sample. Assessment of whether a PCR band contains a gene variant may be carried out in a number of ways familiar to those skilled in the art. The PCR product may for instance be treated in a way that enables one to display the mutation or polymorphism on a denaturing polyacrylamide DNA sequencing gel, with specific bands that are linked to the gene variants being selected.

An alternative or supplement to looking for the presence of variant sequences in a test sample is to look for the presence of the normal sequence, e.g. using a suitably specific oligonucleotide probe or primer.

Approaches which rely on hybridisation between a probe and test nucleic acid and subsequent detection of a mismatch may be employed. Under appropriate conditions (temperature, pH etc.), an oligonucleotide probe will hybridise with a sequence which is not entirely complementary. The degree of base-pairing between the two molecules will be sufficient for them to anneal despite a mis-match. Various approaches are well known in the art for detecting the presence of a mis-match between two annealing nucleic acid molecules.

For instance, RN'ase A cleaves at the site of a mis-match. Cleavage can be detected by electrophoresing test nucleic acid to which the relevant probe or probe has annealed and looking for smaller molecules (i.e. molecules with higher electrophoretic mobility) than the full length probe/test hybrid. Other approaches rely on the use of enzymes such as resolvases or endonucleases.

Thus, an oligonucleotide probe that has the sequence of a region of the normal gene (either sense or anti-sense strand) in which mutations associated with disease resistance are known to occur (e.g. see Table 1) may be annealed to test nucleic acid and the presence or absence of a mis-match determined. Detection of the presence of a mis-match may indicate the presence in the test nucleic acid of a mutation associated with disease resistance. On the other hand, an oligonucleotide probe that has the sequence of a region of the gene including a mutation associated with disease resistance may be annealed to test nucleic acid and the presence or absence of a mis-match determined. The presence of a mis-match may indicate that the nucleic acid in the test sample has the normal sequence, or a different mutant or allele sequence. In either case, a battery of probes to different regions of the gene may be employed.

The presence of differences in sequence of nucleic acid molecules may be detected by means of restriction enzyme digestion, such as in a method of DNA fingerprinting where the restriction pattern produced when one or more restriction enzymes are used to cut a sample of nucleic acid is compared

with the pattern obtained when a sample containing the normal gene or a variant or allele is digested with the same enzyme or enzymes.

The presence of absence of a lesion in a promoter or other regulatory sequence may also be assessed by determining the level of mRNA production by transcription or the level of polypeptide production by translation from the mRNA.

Nucleic acid isolated and/or purified from one or more cells of a plant or a nucleic acid library derived from nucleic acid isolated and/or purified from cells (e.g. a cDNA library derived from mRNA isolated from the cells), may be probed under conditions for selective hybridisation and/or subjected to a specific nucleic acid amplification reaction such as the polymerase chain reaction (PCR).

A method may include hybridisation of one or more (e.g. two) probes or primers to target nucleic acid. Where the nucleic acid is double-stranded DNA, hybridisation will generally be preceded by denaturation to produce single-stranded DNA. The hybridisation may be as part of a PCR procedure, or as part of a probing procedure not involving PCR. An example procedure would be a combination of PCR and low stringency hybridisation. A screening procedure, chosen from the many available to those skilled in the art, is used to identify successful hybridisation events and isolate hybridised nucleic acid.

Binding of a probe to target nucleic acid (e.g. DNA) may be measured using any of a variety of techniques at the

disposal of those skilled in the art. For instance, probes may be radioactively, fluorescently or enzymatically labelled. Other methods not employing labelling of probe include examination of restriction fragment length polymorphisms, amplification using PCR, RNAase cleavage and allele specific oligonucleotide probing.

Probing may employ the standard Southern blotting technique. For instance DNA may be extracted from cells and digested with different restriction enzymes. Restriction fragments may then be separated by electrophoresis on an agarose gel, before denaturation and transfer to a nitrocellulose filter. Labelled probe may be hybridised to the DNA fragments on the filter and binding determined. DNA for probing may be prepared from RNA preparations from cells.

Preliminary experiments may be performed by hybridising under low stringency conditions various probes to Southern blots of DNA digested with restriction enzymes. Suitable conditions would be achieved when a large number of hybridising fragments were obtained while the background hybridisation was low. Using these conditions nucleic acid libraries, e.g. cDNA libraries representative of expressed sequences, may be searched.

As noted, those skilled in the art are well able to employ suitable conditions of the desired stringency for selective hybridisation, taking into account factors such as oligonucleotide length and base composition, temperature and so on.

In some preferred embodiments of diagnostic assays according to the present invention, oligonucleotides according to the present invention that are fragments of any of the sequences shown in Figure 2, or any allele associated with disease resistance, e.g. as identified in Table 1, are at least about 10 nucleotides in length, more preferably at least about 15 nucleotides in length, more preferably at least about 20 nucleotides in length, more preferably about 30 nucleotides in length. Such fragments themselves individually represent aspects of the present invention. Fragments and other oligonucleotides may be used as primers or probes as discussed but may also be generated (e.g. by PCR) in methods concerned with determining the presence in a test sample of a sequence indicative of disease resistance.

There are various methods for determining the presence or absence in a test sample of a particular polypeptide, such as the polypeptide with the amino acid sequence shown in Figure 2, or other figure herein, or an amino acid sequence mutant, variant or allele thereof (e.g. including an alteration shown in Table 1).

A sample may be tested for the presence of a binding partner for a specific binding member such as an antibody (or mixture of antibodies), specific for one or more particular variants of the polypeptide shown in Figure 2, e.g. see Table 1.

In such cases, the sample may be tested by being contacted

with a specific binding member such as an antibody under appropriate conditions for specific binding, before binding is determined, for instance using a reporter system as discussed. Where a panel of antibodies is used, different reporting labels may be employed for each antibody so that binding of each can be determined.

A specific binding member such as an antibody may be used to isolate and/or purify its binding partner polypeptide from a test sample, to allow for sequence and/or biochemical analysis of the polypeptide to determine whether it has the sequence and/or properties of the wild-type polypeptide or a particular mutant, variant or allele thereof. Amino acid sequence is routine in the art using automated sequencing machines.

The use of diagnostic tests for mlo alleles allows the researcher or plant breeder to establish, with full confidence and independent from time consuming resistance tests, whether or not a desired allele is present in the plant of interest (or a cell thereof), whether the plant is a representative of a collection of other genetically identical plants (e.g. an inbred variety or cultivar) or one individual in a sample of related (e.g. breeders' selection) or unrelated plants. The *mlo* alleles conferring the desirable disease resistance phenotype are recessive, and are not therefore detectable at the whole plant phenotype level when in a heterozygous condition in the presence of a wild-type Mlo allele. Phenotypic screening for the presence of such recessive alleles

is therefore only possible on material homozygous for the *mlo* locus and so delays substantially the generation in a plant breeding programme at which selection can be reliably and cost-effectively applied. In a backcross breeding programme where, for example, a breeder is aiming to introgress a desirable *mlo* allele into an elite adapted high performing target genotype, the *mlo* locus will be permanently in the heterozygous condition until selfing is carried out. Nucleic acid or polypeptide testing for the presence of the recessive allele avoids the need to test selfed progeny of backcross generation individuals, thus saving considerable time and money. In other types of breeding scheme based on selection and selfing of desirable individuals, nucleic acid or polypeptide diagnostics for the desirable *mlo* alles in high throughput, low cost assays as provided by this invention, reliable selection for the desirable *mlo* alleles can be made at early generations and on more material than would otherwise be possible. This gain in reliability of selection plus the time saving by being able to test material earlier and without costly resistance phenotype screening is of considerable value in plant breeding.

By way of example for nucleic acid testing, the barley *mlo-5* resistance allele is characterized by a G- to A-nucleotide substitution in the predicted start codon of the *Mlo* gene (Table 1). The mutation may easily be detected by standard PCR amplification of a *Mlo* gene segment from genomic template DNA with the primers:

forward primer: 5'-GTTGCCACACTTTGCCACG-3'

reverse primer: 5'-AAGCCAAGACGACAATCAGA-3'

(for example), followed by digestion witht he restriction enzyme *Psh*AI. This generates a cleaved amplified polymorphic sequences (CAPS) marker which may be displayed using conventional agarose gel electrophoresis. Presence of a 769 bp fragment is indicative of the presence of the *mlo-5* allele.

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The *mlo-9* resistance allele is characterized by a C- to T-nucleotide substitution (Table 1). This allele is of particular relevance since it is used frequently in breeding material. The mutational event may be easily detected using the primers:

forward primer 5'-GRRGCCACACTTTGCCACG-3'

reverse primer 5'-AAGCCAAGACGACAATCAGA-3'

(for example) and subsequent digestion of genomic amplification products with the restriction enzyme *Hha*1. This generates a CAPS marker which may be displayed by conventional agarose gel electrophoresis. The presence of a 374 bp fragment is indicative of the presence of *mlo-9*.

A third, particularly interesting allele is *mlo-12*, characterised by a substitution a residue 240, specifically a Phe240 to leucine replacement. This may result from a C720 to A substitution in the encoding nucleotide sequence (Table 1). This is the only currently documented *mlo* allele for which conclusive evidence is available that the altered protein retains residual wild-type activity (Hentrich, 1979, *Arch. Züchtungsvorsch., Berlin* 9, S. 283-291). *mlo-12* exhibits no detectable spontaneous cell death reaction but confers a

sufficient level of resistance to pathogens such as the powdery mildew fungus. *mlo-12* may therefore be the allele of choice in breeding programs if minimal pleiotropic effects (spontaneous cell death) are desirable after introgression of the *mlo* resistance in elite breeding lines. Furthermore, the molecular site of the amino acid substitution within the Mlo protein allows the design of alleles with a residual wild-type activity, and also the obtention of interacting and/or inhibitory molecules, reducing undesirable pleiotropic effects from a complete loss of function of the Mlo protein.

Nucleic acid-based determination of the presence or absence of *mlo* alleles may be combined with determination of the genotype of the flanking linked genomic DNA and other unlinked genomic DNA using established sets of markers such as RFLPs, microsatellites or SSRs, AFLPs, RAPDs etc. This enables the researcher or plant breeder to select for not only the presence of the desirable *mlo* allele but also for individual plant or families of plants which have the most desirable combinations of linked and unlinked genetic background. Such recombinations of desirable material may occur only rarely within a given segregating breeding population or backcross progeny. Direct assay of the *mlo* locus as afforded by the present invention allows the researcher to make a stepwise approach to fixing (making homozygous) the desired combination of flanking markers and mlo alleles, by first identifying individuals fixed for one flanking marker and then identifying progeny fixed on the other side of the mlo locus all the time

knowing with confidence that the desirable *mlo* allele is still present.

The present disclosure provides sufficient information for a person skilled in the art to obtain genomic DNA sequence for any given new or existing *mlo* allele and devise a suitable nucleic acid- and/or polypeptide-based diagnostic assay. Existing *mlo* alleles to which this may be applied include, for example, *mlo*-1, *mlo*-3, *mlo*-4, *mlo*-5, *mlo*-6, *mlo*-7, *mlo*-8, *mlo*-9, *mlo*-10, *mlo*-12, *mlo*-13, *mlo*-16, *mlo*-17, *mlo*-26 and *mlo*-28, for all of which sequence information is provided herein (see e.g. Figure 2 and Table 1). In designing a nucleic acid assay account is taken of the distinctive variation in sequence that characterises the particular variant allele. Thus, the present invention extends to an oligonucleotide fragment of a *mlo* allele, having a sequence which allows it to hybridise specifically to that allele as compared with other *mlo* alleles. Such an oligonucleotide spans a nucleotide at which a *mlo* mutation occurs, and may include the mutated nucleotide at or towards its 3' or 5' end. Such an oligonucleotide may hybridise with the sense or anti-sense strand. The variation may be within the coding sequence of the mlo gene, or may lie within an intron sequence or in an upstream or downstream non-coding sequence, wherein disruption affects or is otherwise related to the lesion in *Mlo* that results in the mildew resistant phenotype.

The *mlo*-9 allele is widely but not exclusively used in plant breeding (J Helms Jorgensen - *Euphytica* (1992) 63: 141-

152), *mlo*-11 is also used. Use of *mlo* mutants in practical breeding has largely been restricted to spring barley, because the spontaneous cell death response associated with many of the mutant alleles appears to represent a penalty to plant growth and performance when incorporated into high yielding winter barley genotypes. However different *mlo* alleles have different degrees of associated spontaneous cell death response, and thus some, either existing or newly created from mutagenesis programmes or isolated as spontaneous mutants, are more suitable than others for incorporation into winter barley backgrounds. The *mlo*-12 allele may be particularly suitable since no detectable pleiotropic effects occur despite conferring a sufficient level of pathogen resistance. The use of mlo based mildew resistance more widely in winter barleys will have significant value for barley growers as well as significant economic and environmental implications such as reduced use of fungicide inputs with their associated treatment costs. The provision of nucleic acid diagnostics as provided herein enables rapid and accurate deployment of new and existing *mlo* alleles into winter barley germplasm.

Plants which include a plant cell according to the invention are also provided, along with any part or propagule thereof, seed, selfed or hybrid progeny and descendants. A plant according to the present invention may be one which does not breed true in one or more properties. Plant varieties may be excluded, particularly registrable plant varieties according

to Plant Breeders' Rights. It is noted that a plant need not be considered a "plant variety" simply because it contains stably within its genome a transgene, introduced into a cell of the plant or an ancestor thereof.

In addition to a plant, the present invention provides any clone of such a plant, seed, selfed or hybrid progeny and descendants, and any part of any of these, such as cuttings, seed. The invention provides any plant propagule, that is any part which may be used in reproduction or propagation, sexual or asexual, including cuttings, seed and so on. Also encompassed by the invention is a plant which is a sexually or asexually propagated off-spring, clone or descendant of such a plant, or any part or propagule of said plant, off-spring, clone or descendant.

A further aspect of the present invention provides a method of making a plant cell involving introduction of the sequence (e.g. as part of a suitable vector) into a plant cell and causing or allowing recombination between the vector and the plant cell genome to introduce the sequence of nucleotides into the genome.

Following transformation of a plant cell a plant may be regenerated.

The invention further provides a method of modulating *Mlo* expression in a plant, which may modulate a defence response in the plant, comprising expression of a heterologous *Mlo* gene sequence (or mutant, allele, variant or homologue thereof, as

discussed) within cells of the plant. As discussed further herein, modulation or alteration of the level of constitutive defence response in a plant may be by way of suppression, repression or reduction (in the manner of wild-type *Mlo*) or promotion, stimulation, activation, increase, enhancement or augmentation (in the manner of mutant *mlo*). Activation or enhancement of the defence response may confer or increase pathogen resistance of the plant, especially resistance to powdery mildew and/or rust (such as yellow rust).

The term "heterologous" may be used to indicate that the gene/sequence of nucleotides in question have been introduced into said cells of the plant or an ancestor thereof, using genetic engineering, ie by human intervention. A transgenic plant cell, i.e. transgenic for the nucleic acid in question, may be provided. The transgene may be on an extra-genomic vector or incorporated, preferably stably, into the genome. A heterologous gene may replace an endogenous equivalent gene, ie one which normally performs the same or a similar function, or the inserted sequence may be additional to the endogenous gene or other sequence. An advantage of introduction of a heterologous gene is the ability to place expression of a sequence under the control of a promoter of choice, in order to be able to influence expression according to preference, such as under particular developmental, spatial or temporal control, or under control of an inducible promoter. Furthermore, mutants, variants and derivatives of the wild-type gene, e.g. with higher or lower activity than wild-type, may be used in

place of the endogenous gene. Nucleic acid heterologous, or exogenous or foreign, to a plant cell may be non-naturally occuring in cells of that type, variety or species. Thus, nucleic acid may include a coding sequence of or derived from a particular type of plant cell or species or variety of plant, placed within the context of a plant cell of a different type or species or variety of plant. A further possibility is for a nucleic acid sequence to be placed within a cell in which it or a homologue is found naturally, but wherein the nucleic acid sequence is linked and/or adjacent to nucleic acid which does not occur naturally within the cell, or cells of that type or species or variety of plant, such as operably linked to one or more regulatory sequences, such as a promoter sequence, for control of expression. A sequence within a plant or other host cell may be identifiably heterologous, exogenous or foreign.

Down-regulation of wild-type *Mlo* gene function leads to stimulation of a constitutive defence response. This may be achieved in a number of different ways, as illustrated below.

The nucleic acid according to the invention may be placed under the control of an inducible gene promoter thus placing expression under the control of the user.

In a further aspect the present invention provides a gene construct comprising an inducible promoter operatively linked to a nucleotide sequence provided by the present invention. As discussed, this enables control of expression of the gene. The invention also provides plants transformed with said gene construct and methods comprising introduction of such a

construct into a plant cell and/or induction of expression of a construct within a plant cell, e.g. by application of a suitable stimulus, such as an effective exogenous inducer or endogenous signal.

The term "inducible" as applied to a promoter is well understood by those skilled in the art. In essence, expression under the control of an inducible promoter is "switched on" or increased in response to an applied stimulus (which may be generated within a cell or provided exogenously). The nature of the stimulus varies between promoters. Some inducible promoters cause little or undetectable levels of expression (or no expression) in the absence of the appropriate stimulus. Other inducible promoters cause detectable constitutive expression in the absence of the stimulus. Whatever the level of expression is in the absence of the stimulus, expression from any inducible promoter is increased in the presence of the correct stimulus. The preferable situation is where the level of expression increases upon application of the relevant stimulus by an amount effective to alter a phenotypic characteristic. Thus an inducible (or "switchable") promoter may be used which causes a basic level of expression in the absence of the stimulus which level is too low to bring about a desired phenotype (and may in fact be zero). Upon application of the stimulus, expression is increased (or switched on) to a level which brings about the desired phenotype.

Suitable promoters include the Cauliflower Mosaic Virus 35S (CaMV 35S) gene promoter that is expressed at a high level

in virtually all plant tissues (Benfey et al, (1990a) EMBO J 9: 1677-1684); the cauliflower meri 5 promoter that is expressed in the vegetative apical meristem as well as several well localised positions in the plant body, eg inner phloem, flower primordia, branching points in root and shoot (Medford, J.I. (1992) *Plant Cell* 4, 1029-1039; Medford *et al*, (1991) *Plant Cell* 3, 359-370) and the *Arabidopsis thaliana* LEAFY promoter that is expressed very early in flower development (Weigel *et al*, (1992) *Cell* 69, 843-859).

An aspect of the present invention is the use of nucleic acid according to the invention in the production of a transgenic plant.

When introducing a chosen gene construct into a cell, certain considerations must be taken into account, well known to those skilled in the art. The nucleic acid to be inserted should be assembled within a construct which contains effective regulatory elements which will drive transcription. There must be available a method of transporting the construct into the cell. Once the construct is within the cell membrane, integration into the endogenous chromosomal material either will or will not occur. Finally, as far as plants are concerned the target cell type must be such that cells can be regenerated into whole plants.

Plants transformed with the DNA segment containing the sequence may be produced by standard techniques which are already known for the genetic manipulation of plants. DNA can be transformed into plant cells using any suitable technology,

such as a disarmed Ti-plasmid vector carried by Agrobacterium exploiting its natural gene transfer ability (EP-A-270355, EP-A-0116718, NAR 12(22) 8711 - 87215 1984), particle or microprojectile bombardment (US 5100792, EP-A-444882, EP-A-434616) microinjection (WO 92/09696, WO 94/00583, EP 331083, EP 175966, Green et al. (1987) *Plant Tissue and Cell Culture*, Academic Press), electroporation (EP 290395, WO 8706614) other forms of direct DNA uptake (DE 4005152, WO 9012096, US 4684611), liposome mediated DNA uptake (e.g. Freeman et al. *Plant Cell Physiol.* 29: 1353 (1984)), or the vortexing method (e.g. Kindle, *PNAS U.S.A.* 87: 1228 (1990d) Physical methods for the transformation of plant cells are reviewed in Oard, 1991, *Biotech. Adv.* 9: 1-11.

Agrobacterium transformation is widely used by those skilled in the art to transform dicotyledonous species. Recently, there has been substantial progress towards the routine production of stable, fertile transgenic plants in almost all economically relevant monocot plants (Toriyama, et al. (1988) *Bio/Technology* 6, 1072-1074; Zhang, et al. (1988) *Plant Cell Rep.* 7, 379-384; Zhang, et al. (1988) *Theor Appl Genet* 76, 835-840; Shimamoto, et al. (1989) *Nature* 338, 274-276; Datta, et al. (1990) *Bio/Technology* 8, 736-740; Christou, et al. (1991) *Bio/Technology* 9, 957-962; Peng, et al. (1991) International Rice Research Institute, Manila, Philippines 563-574; Cao, et al. (1992) *Plant Cell Rep.* 11, 585-591; Li, et al. (1993) *Plant Cell Rep.* 12, 250-255; Rathore, et al. (1993) *Plant Molecular Biology* 21, 871-884; Fromm, et al. (1990)

Bio/Technology 8, 833-839; Gordon-Kamm, *et al.* (1990) *Plant Cell* 2, 603-618; D'Halluin, *et al.* (1992) *Plant Cell* 4, 1495-1505; Walters, *et al.* (1992) *Plant Molecular Biology* 18, 189-200; Koziel, *et al.* (1993) *Biotechnology* 11, 194-200; Vasil, I. K. (1994) *Plant Molecular Biology* 25, 925-937; Weeks, *et al.* (1993) *Plant Physiology* 102, 1077-1084; Somers, *et al.* (1992) *Bio/Technology* 10, 1589-1594; WO92/14828). In particular, *Agrobacterium* mediated transformation is now emerging also as an highly efficient alternative transformation method in monocots (Hiei *et al.* (1994) *The Plant Journal* 6, 271-282).

The generation of fertile transgenic plants has been achieved in the cereals rice, maize, wheat, oat, and barley (reviewed in Shimamoto, K. (1994) *Current Opinion in Biotechnology* 5, 158-162.; Vasil, *et al.* (1992) *Bio/Technology* 10, 667-674; Vain et al., 1995, *Biotechnology Advances* 13 (4): 653-671; Vasil, 1996, *Nature Biotechnology* 14 page 702).

Microprojectile bombardment, electroporation and direct DNA uptake are preferred where Agrobacterium is inefficient or ineffective. Alternatively, a combination of different techniques may be employed to enhance the efficiency of the transformation process, eg bombardment with Agrobacterium coated microparticles (EP-A-486234) or microprojectile bombardment to induce wounding followed by co-cultivation with Agrobacterium (EP-A-486233).

Following transformation, a plant may be regenerated, e.g. from single cells, callus tissue or leaf discs, as is standard in the art. Almost any plant can be entirely regenerated from

cells, tissues and organs of the plant. Available techniques are reviewd in Vasil et al., *Cell Culture and Somatic Cel Genetics of Plants, Vol I, II and III, Laboratory Procedures and Their Applications*, Academic Press, 1984, and Weissbach and Weissbach, *Methods for Plant Molecular Biology*, Academic Press, 1989.

The particular choice of a transformation technology will be determined by its efficiency to transform certain plant species as well as the experience and preference of the person practising the invention with a particular methodology of choice. It will be apparent to the skilled person that the particular choice of a transformation system to introduce nucleic acid into plant cells is not essential tc or a limitation of the invention, nor is the choice of technique for plant regeneration.

In the present invention, expression may be achieved by introduction of the nucleotide sequence in a sense orientation. Thus, the present invention provides a method of modulation of a defence response in a plant, the method comprising causing or allowing expression of nucleic acid according to the invention within cells of the plant. Generally, it will be desirable to stimulate the defence response, and this may be achieved by disrupting *Mlo* gene function.

Down-regulation of expression of a target gene may be achieved using anti-sense technology or "sense regulation" ("co-suppression").

In using anti-sense genes or partial gene sequences to

down-regulate gene expression, a nucleotide sequence is placed under the control of a promoter in a "reverse orientation" such that transcription yields RNA which is complementary to normal mRNA transcribed from the "sense" strand of the target gene. See, for example, Rothstein et al, 1987; Smith *et al*, (1988) *Nature* 334, 724-726; Zhang *et al*, (1992) *The Plant Cell* 4, 1575-1588, English *et al*., (1996) *The Plant Cell* 8, 179-188. Antisense technology is also reviewed in Bourque, (1995), *Plant Science* 105, 125-149, and Flavell, (1994) *PNAS USA* 91, 3490-3496.

An alternative is to use a copy of all or part of the target gene inserted in sense, that is the same, orientation as the target gene, to achieve reduction in expression of the target gene by co-suppression. See, for example, van der Krol et *al*., (1990) *The Plant Cell* 2, 291-299; Napoli et *al*., (1990) *The Plant Cell* 2, 279-289; Zhang *et al*., (1992) *The Plant Cell* 4, 1575-1588, and US-A-5,231,020.

The complete sequence corresponding to the coding sequence (in reverse orientation for anti-sense) need not be used. For example fragments of sufficient length may be used. It is a routine matter for the person skilled in the art to screen fragments of various sizes and from various parts of the coding sequence to optimise the level of anti-sense inhibition. It may be advantageous to include the initiating methionine ATG codon, and perhaps one or more nucleotides upstream of the initiating codon. A further possibility is to target a conserved sequence of a gene, e.g. a sequence that is

characteristic of one or more genes, such as a regulatory sequence. Antisense constructs may involve 3'end or 5'end sequences of *Mlo* or homologues. In cases where several *Mlo* homologues exist in a plant species, the involvement of 5'- and 3'-end untranslated sequences in the construct will enhance specificity of silencing.

The sequence employed may be about 500 nucleotides or less, possibly about 400 nucleotides, about 300 nucleotides, about 200 nucleotides, or about 100 nucleotides. It may be possible to use oligonucleotides of much shorter lengths, 14-23 nucleotides, although longer fragments, and generally even longer than about 500 nucleotides are preferable where possible, such as longer than about 600 nucleotides, than about 700 nucleotides, than about 800 nucleotides, than about 1000 nucleotides, than about 1200 nucleotides, than about 1400 nucleotides, or more.

It may be preferable that there is complete sequence identity in the sequence used for down-regulation of expression of a target sequence, and the target sequence, though total complementarity or similarity of sequence is not essential. One or more nucleotides may differ in the sequence used from the target gene. Thus, a sequence employed in a down-regulation of gene expression in accordance with the present invention may be a wild-type sequence (e.g. gene) selected from those available, or a mutant, derivative, variant or allele, by way of insertion, addition, deletion or substitution of one or more nucleotides, of such a sequence. The sequence need not

include an open reading frame or specify an RNA that would be translatable. It may be preferred for there to be sufficient homology for the respective anti-sense and sense RNA molecules to hybridise. There may be down regulation of gene expression even where there is about 5%, 10%, 15% or 20% or more mismatch between the sequence used and the target gene.

Generally, the transcribed nucleic acid may represent a fragment of an Mlo gene, such as including a nucleotide sequence shown in Figure 2, or the complement thereof, or may be a mutant, derivative, variant or allele thereof, in similar terms as discussed above in relation to alterations being made to a coding sequence and the homology of the altered sequence. The homology may be sufficient for the transcribed anti-sense RNA to hybridise with nucleic acid within cells of the plant, though irrespective of whether hybridisation takes place the desired effect is down-regulation of gene expression.

Anti-sense regulation may itself be regulated by employing an inducible promoter in an appropriate construct.

Constructs may be expressed using the natural promoter, by a constitutively expressed promotor such as the CaMV 35S promotor, by a tissue-specific or cell-type specific promoter, or by a promoter that can be activated by an external signal or agent. The CaMV 35S promoter but also the rice *actin1* and maize ubiquitin promoters have been shown to give high levels of reporter gene expression in rice (Fujimoto *et al.*, (1993) *Bio/Technology* 11, 1151-1155; Zhang, *et al.*, (1991) *Plant Cell* 3, 1155-1165; Cornejo *et al.*, (1993) *Plant Molecular Biology*

23, 567-581).

For use in anti-sense regulation, nucleic acid including a nucleotide sequence complementary to a coding sequence of a *Mlo* gene (i.e. including homologues), or a fragment of a said coding sequence suitable for use in anti-sense regulation of expression, is provided. This may be DNA and under control of an appropriate regulatory sequence for anti-sense transcription in cells of interest.

Thus, the present invention also provides a method of conferring pathogen resistance on a plant, the method including causing or allowing anti-sense transcription from heterologous nucleic acid according to the invention within cells of the plant.

The present invention further provides the use of the nucleotide sequence of Figure 2 or a fragment, mutant, derivative, allele, variant or homologue thereof, such as any sequence shown or identified herein, for down-regulation of gene expression, particularly down-regulation of expression of an Mlo gene or homologue thereof, preferably in order to confer pathogen resistance on a plant.

When additional copies of the target gene are inserted in sense, that is the same, orientation as the target gene, a range of phenotypes is produced which includes individuals where over-expression occurs and some where under-expression of protein from the target gene occurs. When the inserted gene is only part of the endogenous gene the number of under-expressing individuals in the transgenic population increases. The

mechanism by which sense regulation occurs, particularly down-regulation, is not well-understood. However, this technique is well-reported in scientific and patent literature and is used routinely for gene control. See, for example, van der Krol *et al.*, (1990) *The Plant Cell 2*, 291-229; Napoli *et al.*, (1990) The Plant Cell 2, 279-289; Zhang et al, 1992 *The Plant Cell* 4, 1575-1588.

Again, fragments, mutants and so on may be used in similar terms as described above for use in anti-sense regulation.

Thus, the present invention also provides a method of conferring pathogen resistance on a plant, the method including causing or allowing expression from nucleic acid according to the invention within cells of the plant. This may be used to suppress Mlo activity. Here the activity of the product is preferably suppressed as a result of under-expression within the plant cells.

As noted, *Mlo* down-regulation may promote activation of a defence response, which may in turn confer or augment pathogen resistance of the plant, especially resistance to powdery mildew and/or rust (e.g. yellow rust).

Thus, the present invention also provides a method of modulating *Mlo* function in a plant, the method comprising causing or allowing expression from nucleic acid according to the invention within cells of the plant to suppress endogenous *Mlo* expression.

Modified versions of *Mlo* may be used to down-regulate endogenous *Mlo* function. For example mutants, variants,

derivatives etc., may be employed. For instance, expression of a *mlo* mutant sequence at a high level may out-compete activity of endogenous *Mlo*.

Reduction of *Mlo* wild type activity may be achieved by using ribozymes, such as replication ribozymes, e.g. of the hammerhead class (Haseloff and Gerlach, 1988, *Nature* 334: 585-591; Feyter *et al. Mol.*, 1996, *Gen. Genet.* 250: 329-338).

Another way to reduce *Mlo* function in a plant employs transposon mutagenesis (reviewed by Osborne *et al.*, (1995) *Current Opinion in Cell Biology* 7, 406-413). Inactivation of genes has been demonstrated via a 'targeted tagging' approach using either endogenous mobile elements or heterologous cloned transposons which retain their mobility in alien genomes. *Mlo* alleles carrying any insertion of known sequence could be identified by using PCR primers with binding specificities both in the insertion sequence and the *Mlo* homologue. 'Two-element systems' could be used to stabilize the transposon within inactivated alleles. In the two-element approach, a T-DNA is constructed bearing a non-autonomous transposon containing selectable or screenable marker gene inserted into an excision marker. Plants bearing these T-DNAs are crossed to plants bearing a second T-DNA expressing transposase function. Hybrids are double-selected for excision and for the marker within the transposon yielding F_2 plants with transposed elements. The two-element approach has a particular advantage with respect to *Ac/Ds* of maize, as the transposed *Ds* is likely to be unlinked to the transposase, facilitating outcrossing and stabilization

of the *Ds* insertion (Jones *et al.*,(1994) *Science* 266, 789-793; Osborne *et al.*, (1995) *Current Opinion in Cell Biology* 7, 406-413).

The *mlo*-based powdery mildew resistance is caused by the inactivation of the *Mlo* wild type allele, resulting in a recessive resistance phenotype. Substances that inhibit the activity of the Mlo wild type protein may be used to induce the resistance phenotype.

An important hint that complete inactivation of *Mlo* expression is not essential and may even be detrimental is provided by the description of mutagen-induced *mlo* resistance alleles that are likely to have retained residual wild type allele activity. These alleles exhibit no detectable spontaneous leaf necrosis which negatively affects photosynthesis rates and yield (Hentrich, W (1979) *Arch. Züchtungsvorsch., Berlin* 9, S. 283-291).

The Mlo protein is predicted to be membrane-anchored by seven transmembrane helices (see e.g. Figure 7). This structure prediction has been reinforced by recent analysis of Mlo homologues in rice and *Arabidopsis thaliana*. Structure prediction of the *Arabidopsis thaliana* homologue also suggests the presence of seven transmembrane helices. A comparison of the *Mlo* homologues revealed in addition conserved cysteine residues in the putative extracellular loops 1 and 3 and high probabilities of amphipathic helices in the second intracellular loop adjacent to the predicted transmembrane

helices 3 and 4. These conserved structural motifs in the family of Mlo proteins are reminiscent of G protein coupled receptors (GPCR) described extensively in mammalian systems. GPCRs are known to be activated by ligands and to amplify signals intracellularly via heterotrimeric G proteins. Without in any way providing a limitation on the nature or scope of any aspect of the present invention, it is predicted that Mlo activates an inhibitory G alpha subunit of heterotrimeric G proteins, thus leading to a downregulation of as yet unknown effector proteins.

The provision herein of Mlo sequence information enables the identification of antagonists of function of the Mlo protein (e.g. GPCR function). Antagonists of Mlo may block receptor activation by its unknown genuine ligand, mimicking recessive mutations in the Mlo gene. Such Mlo antagonists may be used as crop protection compounds, for example applied externally to the plant or crop or, where the compound is peptidyl in nature, delivered internally via a biological vector (e.g. recombinant infecting viral particle expressing the antagonistic molecule within target plant cells) or via a transgenic route (plants or plant cells genetically modified to express the antagonist molecule, perhaps under control of a promoter inducible by an externally applied compound (eg GST-II promoter from maize - Jepson et al Plant Molecular Biology 26:1855-1866 (1994)) allowing control over the timing of expresion of the mlo inactivation phenotype.

Leaf segments of *Mlo* wild type plants may be tested with a

test substance, e.g. from a random or combinatorial compound library, for resistance upon challenge with pathogen such as powdery mildew. The detached leaf segment assay is used as a standard test system to score for susceptibility/resistance upon inoculation with powdery mildew spores. Leaf segments of 7-day-old seedlings of the genotype *Mlo RorI* may be placed on agar, for example individual wells of 96-well microtiter plates containing 50μl agar. Different compounds may be applied to the agar surface in each well at a concentration of about 1ppm dissolved in DMSO. Around seven days after inoculation of the detached leaf segments with pathogen, such as spores of a virulent powdery mildew isolate, compounds which induce resistance may be recognised by the absence of fungal mycelium on leaf segments in the microtiter plates.

A further selection may be used to discriminate between compounds that act in the mlo pathway and those that confer resistance by other mechanisms, or those which exhibit a direct fungitoxic activity. For this purpose mutants in genes (*Ror* genes) which may be required for *mlo* resistance (Freialdenhoven et al., (1996), The Plant Cell 8, 5-14) may be used. Mutants of these genes confer <u>susceptibility</u> to powdery mildew attack despite the presence of <u>mlo</u> resistance alleles. Plants of the genotype *Mlo rorl* (wild type *Mlo* protein and defective *Rorl* gene) may be used, for example, to test compounds which induce resistance on *Mlo Rorl* genotypes but exhibit susceptibility on the *Mlo rorl* genotype, enabling selection of candidate *Mlo* antagonists. Testing candidate compounds identified using a

leaf segment test may be used to drastically reduce the number of candidate compounds for further *in vitro* tests.

A further selection step of candidate antagonists may involve heterologous expression of the Mlo protein or a fragment thereof (e.g. in a baculovirus insect cell system) and subsequent binding assays with labelled molecules. Specific binding of compounds to cell lines expressing wild type Mlo protein is a good indicator of their antagonistic mode of action. Analsis of the deduced Mlo protein sequence has provided strong evidence that the protein is anchored in the membrane via seven transmembrane helices and may represent a novel member of the so-called serpentine receptor family. The conclusion is supported by the sequence data derived from homologous genes identified in barley, rice and *Arabidopsis*. Seven transmembrane proteins have been shown to be expressed at high level in the Baculovirus/insect cell system (up to 10^7 molecules per cell - Tate and Grisshamer, 1996, *TIBTECH* 14: 426-430). Since the family of Mlo proteins appears to be restricted to the plant kingdom, this provides a low-background environment for compound tests. Candidate compounds which are labelled, radioactively or non-radioactively, may be tested for specific binding to Sf9 insect cells expressing the Mlo protein after infecion with a recombinant baculovirus construct. Specificity of the binding may be tested further by Sf9 expression of mutant *mlo* proteins which carry characterised mutations (e.g. as in Table 1) leading *in vivo* to resistance.

Thus, in various further aspects the present invention relates to assays for substances able to interfere with Mlo function, i.e. confer a *mlo* mutant phenotype, such substances themselves and uses thereof.

The use of Mlo in identifying and/or obtaining a substance which inhibits Mlo function is further provided by the present invention, as is the use of Mlo in identifying and/or obtaining a substance which induces pathogen resistance in a plant.

Agents useful in accordance with the present invention may be identified by screening techniques which involve determining whether an agent under test inhibits or disrupts Mlo function to induce an *mlo* phenotype. Candidate inhibitors are substances which bind Mlo.

It should of course be noted that references to "Mlo" in relation to assays and screens should be taken to refer to homologues, such as in other species, including rice and wheat, not just in barley, also appropriate fragments, variants, alleles and derivatives thereof. Assessment of whether a test substance is able to bind the Mlo protein does not necessarily require the use of full-length Mlo protein. A suitable fragment may be used (or a suitable analogue or variant thereof).

Suitable fragments of Mlo include those which include residues known to be crucial for Mlo function as identified by mlo mutant alleles (Table 1). Smaller fragments, and analogues and variants of this fragment may similarly be employed, e.g.

as identified using techniques such as deletion analysis or alanine scanning.

Furthermore, one class of agents that can be used to disrupt Mlo activity are peptides fragments of it. Such peptides tend to be short, and may be about 40 amino acids in length or less, preferably about 35 amino acids in length or less, more preferably about 30 amino acids in length, or less, more preferably about 25 amino acids or less, more preferably about 20 amino acids or less, more preferably about 15 amino acids or less, more preferably about 10 amino acids or less, or 9, 8, 7, 6, 5 or less in length. The present invention also encompasses peptides which are sequence variants or derivatives of a wild type Mlo sequence, but which retain ability to interfere with Mlo function, e.g. to induce an *mlo* mutant phenotype. Where one or more additional amino acids are included, such amino acids may be from Mlo or may be heterologous or foreign to Mlo. A peptide may also be included within a larger fusion protein, particularly where the peptide is fused to a non-Mlo(i.e. heterologous or foreign) sequence, such as a polypeptide or protein domain.

Peptides may be generated wholly or partly by chemical synthesis. The compounds of the present invention can be readily prepared according to well-established, standard liquid or, preferably, solid-phase peptide synthesis methods, general descriptions of which are broadly available (see, for example, in J.M. Stewart and J.D. Young, Solid Phase Peptide Synthesis, 2nd edition, Pierce Chemical Company, Rockford, Illinois

1984), in M. Bodanzsky and A. Bodanzsky, The Practice of Peptide Synthesis, Springer Verlag, New York (1984); and Applied Biosystems 430A Users Manual, ABI Inc., Foster City, California), or they may be prepared in solution, by the liquid phase method or by any combination of solid-phase, liquid phase and solution chemistry, e.g. by first completing the respective peptide portion and then, if desired and appropriate, after removal of any protecting groups being present, by introduction of the residue X by reaction of the respective carbonic or sulfonic acid or a reactive derivative thereof.

Another convenient way of producing a peptidyl molecule according to the present invention (peptide or polypeptide) is to express nucleic acid encoding it, by use of nucleic acid in an expression system, as discussed elsewhere herein. This allows for peptide agents to be delivered to plants transgenically, by means of encoding nucleic acid. If coupled to an inducible promoter for expression under control of the user, this allows for flexibility in induction of an *mlo* phenotype and pathogen resistance. This may allow for any side-effects arising from interference with Mlo function to be moderated.

In one general aspect the present invention provides an assay method for a substance able to interact with the relevant region of Mlo, the method including:

(a) bringing into contact a Mlo polypeptide or peptide fragment thereoof, or a variant, derivative or analogue

thereof, and a test compound; and

(b) determining interaction or binding between said polypeptide or peptide and the test compound.

A test compound found to interact with the relevant portion of Mlo may be tested for ability to modulate, e.g. disrupt or interfere with, Mlo function, as discussed already above.

Another general aspect of the present invention provides an assay method for a substance able to induce an *mlo* mutant phenotype in a plant, the method including:

(a) bringing into contact a plant or part thereof (e.g. leaf or leaf segment) and a test compound; and

(b) determining Mlo function and/or pathogen resistance and/or stimulation of a defence response in the plant.

Susceptibility or resistance to a pathogen may be determined by assessing pathogen growth, e.g. for powdery mildew the presence or absence, or extent, of mycelial growth.

Binding of a test compound to a polypeptide or peptide may be assessed in addition to ability of the test compound to stimulate a defence response in a plant. Such tests may be run in parallel or one test may be performed on a substance which tests positive in another test.

Of course, the person skilled in the art will design any appropriate control experiments with which to compare results obtained in test assays.

Performance of an assay method according to the present invention may be followed by isolation and/or manufacture and/or use of a compound, substance or molecule which tests positive for ability to modulate *Mlo* function and/or induce pathogen resistance, such as resistance to powdery mildew.

The precise format of an assay of the invention may be varied by those of skill in the art using routine skill and knowledge. For example, interaction between substances may be studied *in vitro* by labelling one with a detectable label and bringing it into contact with the other which has been immobilised on a solid support. Suitable detectable labels, especially for peptidyl substances include ^{35}S-methionine which may be incorporated into recombinantly produced peptides and polypeptides. Recombinantly produced peptides and polypeptides may also be expressed as a fusion protein containing an epitope which can be labelled with an antibody.

An assay according to the present invention may also take the form of an *in vivo* assay. The *in vivo* assay may be performed in a cell line such as a yeast strain or mammalian cell line in which the relevant polypeptides or peptides are expressed from one or more vectors introduced into the cell.

For example, a polypeptide or peptide containing a fragment of Mlo or a peptidyl analogue or variant thereof as disclosed, may be fused to a DNA binding domain such as that of the yeast transcription factor GAL 4. The GAL 4 transcription factor includes two functional domains. These domains are the

DNA binding domain (GAL4DBD) and the GAL4 transcriptional activation domain (GAL4TAD). By fusing such a polypeptide or peptide to one of those domains and another polypeptide or peptide to the respective counterpart, a functional GAL 4 transcription factor is restored only when two polypeptides or peptides of interest interact. Thus, interaction of the polypeptides or peptides may be measured by the use of a reporter gene probably linked to a GAL 4 DNA binding site which is capable of activating transcription of said reporter gene. This assay format is described by Fields and Song, 1989, Nature 340; 245-246. This type of assay format can be used in both mammalian cells and in yeast. Other combinations of DNA binding domain and transcriptional activation domain are available in the art and may be preferred, such as the LexA DNA binding domain and the VP60 transcriptional activation domain.

When looking for peptides or other substances which interact with Mlo, the Mlo polypeptide or peptide may be employed as a fusion with (e.g.) the LexA DNA binding domain, with test polypeptide or peptide (e.g. a random or combinatorial peptide library) as a fusion with (e.g.) VP60. An increase in reporter gene expression (e.g. in the case of β-galactosidase a strengthening of the blue colour) results from the presence of a peptide which interacts with Mlo, which interaction is required for transcriptional activation of the β-galactosidase gene.

The amount of test substance or compound which may be

added to an assay of the invention will normally be determined by trial and error depending upon the type of compound used. Typically, from about 0.001 nM to 1mM or more concentrations of putative inhibitor compound may be used, for example from 0.01 nM to 100μM, e.g. 0.1 to 50 μM, such as about 10 μM. Greater concentrations may be used when a peptide is the test substance. Even a molecule which has a weak effect may be a useful lead compound for further investigation and development.

Compounds which may be used may be natural or synthetic chemical compounds used in drug screening programmes. Extracts of plants which contain several characterised or uncharacterised components may also be used. Antibodies directed to Mlo or a fragment thereof form a further class of putative inhibitor compounds. Candidate inhibitor antibodies may be characterised and their binding regions determined to provide single chain antibodies and fragments thereof which are responsible for disrupting the interaction. Other candidate inhibitor compounds may be based on modelling the 3-dimensional structure of a polypeptide or peptide fragment and using rational drug design to provide potential inhibitor compounds with particular molecular shape, size and charge characteristics. It is worth noting, however, that combinatorial library technology provides an efficient way of testing a potentially vast number of different substances for ability to interact with and/or modulate the activity of a polypeptide. Such libraries and their use are known in the art, for all manner of natural products, small molecules and

peptides, among others. The use of peptide libraries may be preferred in certain circumstances.

Following identification of a substance or agent which modulates or affects *Mlo* function, the substance or agent may be investigated further. Furthermore, it may be manufactured and/or used in preparation, i.e. manufacture or formulation, of a composition for inducing pathogen resistance in a plant. These may be applied to plants, e.g. for inducing pathogen resistance, such as resistance to powedery mildew. A further aspect of the present invention provides a method of inducing pathogen resistance in a plant, the method including applying such a substance to the plant. A peptidyl molecule may be applied to a plant transgenically, by expression from encoding nucleic acid, as noted.

A polypeptide, peptide or other substance able to modulate or interfere with *Mlo* function, inducing pathogen resistance in a plant as disclosed herein, or a nucleic acid molecule encoding a peptidyl such molecule, may be provided in a kit, e.g. sealed in a suitable container which protects its contents from the external environment. Such a kit may include instructions for use.

Further aspects and embodiments of the present invention will be apparent to those skilled in the art. The present invention will now be exemplified by way of illustration with

reference to the following figures:

Brief Description of the Drawings

Figure 1 Positional Cloning of *Mlo*. The *Mlo* locus has been mapped with increasing precision on the long arm of barley chromosome 4 using morphological, RFLP and AFLP markers. The upper part of the figure presents the genetic linkage maps of these markers relative to *Mlo*. All genetic distances are indicated in centiMorgan (cM) based on multi-point linkage analysis except for genetic distances between AFLP markers which are calculated by two-point-estimates. The morphological marker map (Jørgensen, 1977) positions *Mlo* at a distance of more than 20 cM to hairy leaf sheath (*Hs*) and glossy sheath/spike (*gsl*). The RFLP marker map is based on the analysis of 257 F_2 individuals derived from the cross Carlsberg II *Mlo* Grannenlose Zweizeilige *mlo-11*. The previously published RFLP map (Hinze *et al.*, 1991) of the same cross was based on only 44 F_2 individuals. The gene was delimited to a 2.7 cM interval bordered by markers bAO11 and bAL88. AFLP markers were identified and mapped as described in Experimental procedures. Their genetic distance to *Mlo* is based on the cross Ingrid *Mlo* x BC_7Ingrid *mlo-3*. The crucial result of the AFLP analysis has been the identification of two markers, Bpm2 and Bpm9, defining an 0.64 cM interval containing the *Mlo* locus and one marker (Bpm16) cosegregating with *Mlo* on the basis of more than 4,000 meiotic events. Marker Bxm2 which is located 0.1 cM telomeric to *Mlo* was derived from BAC F15 template DNA (see below). One YAC clone, YAC YHV303-A6, containing the

cosegregating marker Bpm16 and two flanking loci (Bpm2 and Bpm9), is shown in the middle section of the figure. The position of marker Bpm9 was only roughly estimated within the YAC clone as indicated by the arrow. The insert of BAC F15 represents a 60 kb subfragment of this YAC as indicated in the lower part of the Figure. After the identification of AFLP marker Bpm2 in BAC F15, marker Bxm2 was discovered and positioned 0.1 cM in telomeric orientation of *Mlo*. The approximate physical position of AFLP markers Bpm2, Bpm16, and Bxm2 (spanning an interval of approximately 30 kb) as well as the location of some rare occurring restriction sites are indicated. Dashed lines below the schematic representation of BAC F15 DNA show the position of the largest established DNA sequence contigs. The structure of the *Mlo* gene is given schematically in the bottom line of the Figure. Exons are highlighted by black boxes. Positions of mutational events are indicated for the eleven tested *mlo* alleles. Mutant alleles carrying deletions in their nucleotide sequence are marked with a Δ; the remaining mutant alleles represent single nucleotide substitutions resulting in amino acid exchanges in each case.

Sub D4 Figure 2 shows an *Mlo* coding sequence and encoded amino acid sequence according to the present invention. The amino acid sequence predicted from DNA sequences of RT-PCR products from Ingrid *Mlo* are shown. Nucleotide numbers are given according to translational start site.

Figure 3 Northern Blot Analysis of *Mlo* Transcript Accumulation. Total RNA (20 μg) and poly(A)$^+$ RNA (5 μg) of

seven-day-old uninfected barley primary leaves of one wild type (cultivar Ingrid *Mlo*) and two mutant (BC Ingrid *mlo-1*, BC Ingrid *mlo-3*) cultivars were isolated, separated on a 1.2% formaldehyde gel and transferred to a nitrocellulose membrane (Hybond). The filter was probed under stringent conditions (Sambrook *et al.*, 1989) with the radioactivity labelled full size RT-PCR product derived from Ingrid *Mlo* (Figure 7). A clear signal is detected only in the lanes containing poly(A)$^+$ RNA. The signal corresponds to a size of approximately 2 kb.

Figure 4 Southern Blot Analysis of Intragenic Recombinants derived from *mlo* heteroallelic crosses. The alleles of two RFLP markers flanking *Mlo* on opposite sides of either susceptible F_2 individuals or homozygous susceptible and homozygous resistant progeny were determined by Southern blot analysis. Plant DNA (10 μg) of the individuals were digested with *Pst* I (A) or *Hae* III (B) and hybridized with the radioactively labelled RFLP markers WG114 (upper panel; maps 3.1 cM in centromeric orientation to *Mlo*; see Figure 1) and ABG366 (lower panel; maps 0.7 cM in telomeric orientation to *Mlo*; see Figure 1) according to standard procedures (Sambrook *et al.*, 1989).

A DNA of the parental lines *mlo-8* and *mlo-1* and two homozygous susceptible (S, *Mlo Mlo*) and two resistant (R, *mlo mlo*) progenies derived from two susceptible F_2 plants (designated 1 and 2) were tested. The DNAs in lanes S and R represent selection F_3 individuals from F_3 families obtained by selfing the susceptible F_2 individuals 1 and 2. Note that

susceptible F_2 individuals are expected to be heterozygous at *Mlo* in this section scheme. Infection phenotypes were scored seven days after inoculation with the *mlo* avirulent isolate K1. DNA from a third susceptible individual of this heteroallelic cross (see Table 7) is not included in this Figure.

B DNA of the parental lines *mlo-5* and *mlo-1* and seven homozygous susceptible (S, *Mlo Mlo*) and seven resistant (R, *mlo mlo*) progeny derived from seven susceptible F_2 plants (designated 1 to 7) were tested. The DNAs in lanes S and R represent selected F_3 individuals from F_3 families obtained by selfing the susceptible F_2 individuals 1 to 7. DNA was analyzed from two further susceptible individuals of this heteroallelic cross only in the F_2 generation (8* and 9*).

Figure 5 shows an alignment of genomic sequences covering the barley *Mlo* gene and a rice homologue isolated *via* crosshybridization with a barley gene specific probe. The top line shows the barley *Mlo* genomic DNA sequence (exon sequences underlined). The bottom line shows the rice genomic sequence containing the rice *Mlo* homologue.

Figure 6 shows an alignment of genomic sequences carrying the barley *Mlo* gene and a barley homologue isolated *via* crosshybridization with a barley gene specific probe. The top line shows the barley *Mlo* genomic DNA sequence (exon sequences underlined). The bottom line shows the genomic sequence containing the barley *Mlo* homologue.

Figure 7 Nucleotide and Deduced Amino Acid Sequence of the Barley *Mlo* cDNA. The nucleotide and the deduced amino acid

sequence are based on the combined data of RT-PCR and RACE obtained from experiments using RNA of cultivar Ingrid *Mlo*. The stop codon is marked by an asterisk, the putative polyadenylation signal is underlined and the detected termini of RACE products are indicated by arrows above the sequence. Positions of introns as indentified by comparison with corresponding genomic clones are labelled by triangles below the nucleic acid sequence. Six predicted transmembrane spanning helices according to the MEMSAT algorithm (Jones *et al.*, 1994) are boxed in grey colour. A putative nuclear localization signal (K-K-K-V-R) and casein kinase II site (S-I-F-D) in the carboxy-terminal half of the protein are shown in bold type.

Figure 8 shows genomic sequence of rice (Oryza sativa) homologue including coding and flanking sequences.

Figure 9 shows genomic sequence of barley (Hordeum vulgare) homologue including coding and flanking sequences.

Figure 10 shows cDNA sequence of rice homologue.

Figure 11 shows cDNA sequence of barley homologue.

Figure 12 shows cDNA sequence of Arabidopsis thaliana homologue.

Figure 13 shows amino acid sequence of rice homologue.

Figure 14 shows amino acid sequence of barley homologue.

Figure 15 shows amino acid sequence of Arabidopsis homologue.

Figure 16 shows a pretty box of amino acid sequences of Mlo, barley, rice and Arabidopsis homologues.

All documents mentioned in this document are incorporated by reference.

EXAMPLE 1 - CLONING OF MLO OF BARLEY

Targeted search for AFLP markers tightly linked to Mlo

Efforts to increase the DNA marker density around *Mlo* were coordinated with attempts to construct a local high resolution genetic map. An alternative possibility would have been to extend the population size of the characterized cross Carlsberg II *Mlo* x Grannenlose Zweizeilige *mlo-11* (Hinze et al., 1991) but it was felt to be advantageous to establish a high resolution map starting out from one of the available BC *mlo* lines and its recurrent parent line. Importantly, the donor parent of the BC line represents a different genetic background in comparison to the recurrent parent line. In this way, searching for linked AFLP markers could be started in parallel with generating a large mapping population from a cross between the same genetic lines. In addition, the BC line based cross allowed testing of colinearity of DNA markers in the vicinity of *Mlo* as determined from the cross Carlsberg II *Mlo* x Grannenlose Zweizeilige *mlo-11* (Hinze *et al.*, 1991). For the new cross a *mlo-3* backcross (BC) line was used that had been backcrossed seven times into the genetic background Ingrid (BC, Ingrid *mlo-3* ; Hinze *et al.*, 1991). The line was previously characterized to carry a relatively small introgressed DNA segment on barley chromosome 4. In addition,

the donor parent line Malteria Heda *mlo-3* exhibits in comparison to DNA from the recurrent parent Ingrid polymorphisms with most of the identified RFLP loci linked to *Mlo*. Thus, by searching polymorphisms only between two DNA templates, from lines Ingrid *Mlo* and BC_7 Ingrid *mlo-3*, we hoped to increase the density of DNA markers with AFLPs around *Mlo* in a targeted manner.

The same two lines were crossed to establish a segregating population for high resolution mapping of DNA markers, formally representing an eigth backcross. F_2 individuals were scored for *mlo* resistance after powdery mildew inoculation with isolate K1 (virulent on Ingrid *Mlo* and avirulent on BC_7 Ingrid *mlo-3*). Initially, only a small fraction of the F_2 (77 individuals) was analyzed for recombination events with flanking RFLP markers. Analysis of four identified recombinants (designated 8-32-2, 7-38-4, 1-34-1, and 1-49-4) indicated colinearity of marker order in this cross compared to the previously analyzed cross Carlsberg II *Mlo* x Grannenlose Zweizeilige *mlo-11* (Hinze *et al.*, 1991). Several of the 77 F_2 seedlings which exhibited a susceptible phenotype and heterozygosity for the tested flanking DNA marker loci (bAO11, bAL88/2, and bAP91; Hinze *et al.*, 1991) were grown to maturity to provide further selfed seed material segregating for *Mlo/mlo-3* in the F_3 generation. In total, leaf material was harvested for high resolution marker mapping from 2,026 individuals derived from either the selfed F_2 or F_3 generation.

AFLP marker candidates were identified by testing all

possible *Pst* I/*Mse* I primer combinations (1,024) extending into genomic sequences up to nucleotide positions +2 and +3, respectively. Similarly, almost 1,900 *Eco* RI/*Mse* I primer combinations (+3/+3) have been analyzed. Four DNA templates were included in this analysis: Ingrid *Mlo*, BC_7 Ingrid *mlo-3* , a DNA pool of two phenotypically *mlo* resistant F_2 individuals, and a DNA pool of nine phenotypically susceptible F_2 individuals. The resistant and susceptible F_2 individuals which were included as DNA pools in the AFLP search had been selected from the above mentioned RFLP analysis of 77 F_2 segregants. The pooled F_2 DNA enabled us to control whether candidate polymorphisms detected between template DNA from the parents were heritable traits in the F_2. All identified AFLP candidate markers have been re-examined with eight DNA templates: Ingrid *Mlo*, BC_7 Ingrid *mlo-3* , DNA pools from individuals of three F_3 families which were phenotypically homozygous susceptible (*MloMlo*) according to K1 inoculation experiments; DNA of three resistant F_2 individuals. A total of 18 *Pst* I/*Mse* I and 20 *Eco* RI/*Mse* I primers were confirmed based on the selection procedure.

The number of identified AFLP markers made it useful to assign them first roughly to marker intervals based on the RFLP map around *Mlo*. It was hoped that this approach should enable both evaluation of the distribution of AFLPs among previously identified RFLP intervals close to *Mlo* and selection of a pair of flanking AFLP markers with which recombinants could be identified among the 2,026 segregants. For AFLP assignment we

used those four recombinants that had been identified with RFLP markers out of the above mentioned small sample of 77 F_2 segregants from Ingrid *Mlo* x BC_7 Ingrid *mlo-3* (two recombinants in interval bAP91-bAL88, one in *Mlo*-bAO11, and one in bAO11-ABG366). A total of 18 AFLPs were found to be located within a genetic distance of approximately 3.5 cM including *Mlo*.

Construction of a high resolution AFLP map around Mlo

A two-step procedure was used to construct the high resolution AFLP map. First, all 2,026 segregants were screened for recombination events between two AFLP markers on opposite sides of *Mlo* and subsequently only the few identified recombinants were used to map all the identified AFLPs in the 3.5 cM target interval. AFLP markers Bpm1 and Bpm9 were chosen, detecting each allelic DNA fragments in Ingrid *Mlo* and BC_7 Ingrid *mlo-3* and located on opposite sites of *Mlo* to screen DNA templates of the segregants for recombination events. Alternatively, the search for recombinants could have been carried out with the flanking RFLP markers bAO11 and bAL88. However, although the conversion into cleaved amplified polymorphic sites (CAPS) was successful for both markers, difficulties to display the alleles of both loci simultaneously from crudely purified genomic DNA were encountered. A total of 2,026 individuals (F_2 or F_3 segregants) were screened simultaneously with AFLP markers Bpm1 and Bpm9 and 98 recombinants were identified. AFLP analysis was subsequently carried out with each of the 98 DNA templates of the

recombinants to identify the alleles of each of the identified of AFLP loci. The recombinants have been selfed and inoculation experiments with powdery mildew isolate K1 were performed using at least 25 individuals of each recombinant family to deduce the alleles of the previous generation at the *Mlo* locus. The obtained data enabled the construction of a high resolution map around *Mlo* based on more than 4,000 meiotic events and a resolution of at least 0.025 cM derived *via* two-point estimates. The essential result has been the identification of a DNA marker cosegregating with *Mlo* (Bpm16) and two flanking markers (Bpm2 and Bpm9) at a distance of 0.25 and 0.4 cM respectively (Figure 1).

Construction of a large insert size barley YAC library, isolation of Bpm16 containing YACs, and physical delimitation of Mlo

The genetic evidence indicates that *mlo* resistance is due to loss of function in the *Mlo* wild type allele. Therefore, it was decided to establish a large insert size YAC library from cultivar Ingrid *Mlo* into vector pYAC4 (Burke et al., 1987; Hieter, 1990). Megabase DNA suitable for YAC cloning experiments was prepared in mg amounts from mesophyll protoplasts of five-day-old seedlings according to a modified protocol described by Siedler and Graner (1991). The DNA was partially digested with *Eco* RI in the presence of *Eco* RI methyltransferase to obtain DNA fragments after preparative pulsed-field gel electrophoresis (PFGE) in the size range of

500-600 kb. After ligation with *Eco* RI digested pYAC4, the DNA was transformed into yeast strain AB1380 and colonies carrying recombinant pYAC4 DNA were selected on solidified synthetic complete medium lacking tryptophan and uracil (Sherman *et al.*, 1986). Forty randomly selected yeast colonies were tested for the presence of barley DNA using labelled barley genomic DNA in Southern experiments. The size of the YAC inserts was found after PFGE separations to vary between 500 and 800 kb. On average a genetic distance of 0.2 cM was expected to be represented on the individual recombinant YAC clone. A total of ~40,000 clones representing four barley genome equivalents have been generated.

Four YAC clones (designated 303A6, 322G2, 400H11, and 417D1) have been isolated with marker Bpm16 cosegregating with *Mlo*. Their insert size was determined by PFGE to be 650, 710, 650, and 820 kb respectively. AFLP analysis had shown that three of these clones (303A6, 322G2, and 417D1) contain also both flanking marker loci whereas clone 400H11 contains only loci Bpm16 and Bpm2. These findings strongly suggested that the *Mlo* gene had been physically delimited on recombinant YAC clones 303A6, 322G2, and 417D1.

YAC 303A6 was chosen for subcloning experiments into BAC vector pECSBAC4 containing a unique *Eco* RI site (Shizuya *et al.*, 1992; the vector pECSBAC4 is described by Frijters and Michelmore, 1996; submitted). Total yeast DNA of this clone was partially digested with *Eco* RI to obtain DNA fragments with an average size of 50 kb and ligated into *Eco* RI digested and

dephosphorylated BAC vector. Bacterial colonies containing YAC 303A6-derived DNA in pECSBAC4 were identified by replica colony hybridization experiments. One set of colony containing membranes was hybridized with labelled yeast AB1380 DNA and the replica set was hybridized with labelled PFGE-purified YAC303A6 DNA. Recombinant BAC clones containing the AFLP locus Bpm16 were subsequently identified using the cloned 108 bp *Pst* I/*Mse* I genomic Bpm16 fragment as a probe in colony hybridization experiments.

One BAC clone, BAC F15, containing an insert of ~ 60 kb was chosen for further detailed studies. It was found that the recombinant BAC clone contained in addition the AFLP marker locus Bpm2, but not Bpm9. At this point the BAC F15 insert DNA indicated successful physical delimitation in telomeric orientation but it was an open question whether the insert would contain bordering sequences in centromeric direction. Instead of constructing a BAC contig between Bpm 16 and Bpm9, the option to develop new polymorphic markers from BAC F15 was chosen. An allelic *Xba* I/*Mse* I polymorphism (designated Bxm2) was identified between the parental lines Ingrid *Mlo* and BC_7 Ingrid *mlo-3*.

An analysis of the 25 recombinant individuals carrying recombination events within the *Mlo* containing interval Bpm2-Bpm9 enabled mapping of Bxm2 in centrometric orientation at a distance of 0.1 cM from *Mlo*. Only four out of the 16 available recombinants in the interval Bpm9-*Mlo* and none of the 9 recombinants in the interval *Mlo*-Bpm2 were found to exhibit a

recombination event between Bxm2 and *Mlo*. It was concluded that *Mlo* had been physically delimited on BAC F15 between marker loci Bpm2 and Bxm2 (Figure 1).

Identification of the Mlo gene and mlo mutants

A random sequencing project was initiated to determine sequence contigs of the ~60 kb insert of BAC F15 before marker Bxm2 was identified and shown to delimit the gene in telomeric orientation. In parallel, a physical map was generated (Figure 1). The physical map indicated that the flanking markers Bpm2 and Bxm2 are physically separated by ~30 kb. The sequence contigs were searched for regions of high coding probability using the UNIX versions of the STADEN program package. Only one sequence contig of almost 6 kb, including the cosegregating marker Bpm16, revealed an extensive region of high coding probability.

RT-PCR reactions were performed with total leaf RNA derived from cultivar Ingrid *Mlo* using a series of primers deduced from regions which indicated high coding probabilities and obtained in each case a distinct amplification product. Sequencing of the largest RT-PCR products revealed a single extensive open reading frame of 1,602 bp (Figure 2). The deduced putative protein of 533 amino acids has a molecular weight of 60.4 kDal. The ~1.7 kb RT-PCR product was used as a hybridization probe and detected a single RNA transcript of ~1.9 kb length. (Figure 3). A comparison of the genomic sequence and the largest RT-PCR fragment reveals 12 exons and

11 introns, each flanked by the characteristic splice site sequences (Figure 1).

Because marker Bpm16 is located at the 3' end of the above described gene (exon 11) and cosegregates with the *Mlo* locus, we started a direct PCR sequencing of the various available mutagen-induced *mlo* resistance alleles. We identified in 14 out of 15 tested mutant alleles nucleotide alterations which result either in single amino acid alterations, deletions or frame shifts of the wild type sequence (Table 1). We suspect that mutant allele *mlo-2* is located within the promoter- or 5' untranslated sequences. The region is notoriously difficult to be sequenced via direct PCR sequencing from genomic DNA templates but experiments using a series of nested primers are likely to solve this problem. In summary, the comparative sequencing of genomic DNA from various mutant *mlo* lines and their respective *Mlo* wild type ciltivars provided strong evidence that *Mlo* has been identified.

Intragenic recombinants

It had been the intention to provide a chain of evidence for the molecular isolation of *Mlo* which did not rely upon complementation experiments via transgenic barley plants. We had chosen to develop an unusual genetic tool to confirm that the identified gene represented *Mlo*. It was reasoned that if the mutations observed in the above described gene caused resistance to the powdery mildew fungus, recombination events

between mutant allele sites should restore wild type sequences. It was predicted that those intragenic recombinants would exhibit susceptibility upon powdery mildew attack.

A crossing scheme was devised involving mlo resistance alleles mlo-1, mlo-5, and mlo-8. The mutant alleles originate from the genetic backgrounds Haisa (mlo-1) and Carlsberg II (mlo-5 and mlo-8). Intermutant crosses were performed as shown in Table 2 generating in each case at least 10 F_1 plants. F_2 populations were obtained by self-fertilization. F_2 seedlings were screened for rare susceptible individuals after inoculation with powdery mildew isolate K1 which is virulent on each of the parental Mlo wild type cultivars. Susceptible F_2 individuals were identified with a frequency of ~6 x 10^{-4}. In contrast, if comparable numbers of progenies from selfings of each of the *mlo* mutants were tested for resistance to K1, no susceptible seedling was identified. This finding strongly indicated that the majority of the susceptible individuals derived from the intermutant crosses were not due to spontaneous reversion events of the mutant *mlo* alleles.

Inheritance of the susceptible F_2 individuals was tested after selfing in F_3 families. Each of the F2 individuals segregated susceptible and resistant F_3 individuals indicating hetrozygosity for alleles conferring resistance/susceptibility in the F_2. Homozygous susceptible F_3 progeny were isolated for the majority of susceptible F_2 individuals by selfing of F_3 individuals and subsequent identification of F_4 families in which only susceptible individuals were detected.

A molecular analysis of the susceptible individuals has been performed using RFLP markers known to be tightly linked (< 3 cM) on each side of the *Mlo* locus (Figure 4). RFLP marker WG114 maps in centromeric orientation relative to *Mlo*, marker ABG366 maps in the direction of the telomere. Detected RFLP alleles are shown for the intermutant crosses *mlo-8* x *mlo-1* (A) and *mlo-1* x *mlo-5* (B). DNA was analyzed either from susceptible F_2 individuals (indicated by *) or from homozygous susceptible (S) and homozygous resistant (R) F_3 progeny obtained from selfed susceptible F_2 individuals.

The homozygous susceptible F_3 progeny from the susceptible F_2 plant #1 of cross
mlo-8 x *mlo-1* (Figure 4) reveals the WG114 allele derived from the *mlo-1* parent in centromeric orientation next to *Mlo* and the ABG366 allele from the *mlo-8* parent in telomeric orientation to *Mlo*. The homozygous resistant F_3 progeny from F_2 plant #1 of this cross reveals in contrast only the flanking marker alleles derived from parent *mlo-1*. The finding strongly suggested that susceptibility in F_2 plant #1 is caused by a cross-over type of recombination in the preceding meiosis of one chromosome which results in a restoration of the *Mlo* wild type allele whereas the second F_2 chromosome of individual 1 contains a functionally unaltered *mlo-1* allele. The allelotypes of the RFLP loci of the homozygous susceptible F_3 progeny from susceptible F_2 plant #2 are identical to the one described above. However, flanking marker alleles from the homozygous resistant F_3 progeny of this individual are in both cases

derived from the *mlo-8* parent. It is concluded that again a cross-over type of recombination restored one *Mlo* wild type allele in the susceptible F_2 individual #2.

Nine susceptible F_2 individuals were recovered from the cross *mlo-1* x *mlo-5* (Figure 4). For susceptible F_2 individuals #1 to #7 both homozygous susceptible and homozygous resistent F_3 progeny were analyzed at the DNA level. Note that only DNA from the heterozygous susceptible F_2 individuals was analyzed in the case of individuals #8 and #9 (marked by a *). The following allele patterns with respect to the flanking RFLP loci were observed: (*i*) homozygous resistant F_3 progeny showed on both sides of *Mlo* either only the allelotypes of loci WG114 and ABG366 derived from the *mlo-1* parent (individuals #1, #3, #6, #7) or only the allelotypes derived from the *mlo-5* parent (individuals #2, #4, #5). (*ii*) Homozygous susceptible F_3 progeny showed in contrast either only the allelotypes of both loci derived from the *mlo-5* parent (no. #3, #5, #6) or they showed different allelotypes on both sides of *Mlo* (individuals #1, #2, #4, #7). (*iii*) The homozygous susceptible F_3 progeny with different allelotypes on both sides always contain in centromeric orientation the *mlo-1* derived WG114 allele and in telomeric orientation the *mlo-5* derived ABG366 allele. (*iv*) The heterozygous susceptible F_2 individual #8 reveals on either side next to *Mlo* only the alleles derived from parent *mlo-5*. The heterozygous susceptible individual #9 reveals in centromeric orientation alleles derived from both parents *mlo-1* and *mlo-5* whereas only the *mlo-5* derived allele is detected in

telomeric orientation. A comprehensive interpretation of the data suggests that susceptibility in F_2 individuals no. #1, #2, #4, #7, and #9 is caused by a cross-over type of recombination restoring the *Mlo* wild type allele. Non cross-over types of recombination may have restored the *Mlo* wild type allele in individuals no. #3, #5, #6, and #8.

A compilation of the detected flanking RFLP alleles of all isolated susceptible F_2 individuals or homozygous F_3 progeny is shown in Table 3. Note that individual #3 of the cross *mlo-8* x *mlo-1* is not shown in Figure 4. The compilation reveals that (*i*) cross-over types of recombination (CO) and non cross-over types of recombination (NCO) are found with a ratio of 7 : 5, (*ii*) cross-over types of recombination are resolved unidirectional, and (*iii*) NCO recombinants were not observed with parental *mlo-1*-linked RFLP alleles.

The CO type intragenic recombinants isolated from heteroallelic *mlo* crosses were used to test whether wild type sequences of the *Mlo* candidate gene had been restored. For the three relevant alleles *mlo-1*, *mlo-5*, *mlo-8* alleles candidate mutation sites have been identified (Table 1 and 4). Direct PCR sequencing of genomic DNA of susceptible intragenic recombinants derived from both heteroallelic crosses *mlo-1 x mlo-8* and *mlo-1 x mlo-5* revealed restoration of wild type sequences (Table 4). This observation strongly suggests that the intragenic cross over event occurred between nucleotide -1 and +483 in the former and +3 and +483 in the latter cross (according to translational start site). Thus, the molecular

analysis of seven intragenic recombinants from two heteroallelic crosses provides final proof that the above described candidate gene represents *Mlo*.

EXAMPLE 2 - HOMOLOGUES OF THE IDENTIFIED MLO GENE

The available expressed sequence tag (EST) databases of *Oryzae sativa* (rice) and *Arabidopsis thaliana* were searched for homologous protein sequences. Five Arabidopsis cDNA clones were identified whose deduced amino acid sequences show substantial similarity to the Mlo protein. Remarkable is cDNA clone 205N12T7 which reveals a chance probability of 1.2 e^{-45}. In addition, at least one significant homologue was found in rice (OSR16381A).

A rice BAC library (Wang *et al.*, 1995) has also been screened with a labelled barley genomic fragment containing *Mlo*. A BAC clone containing an insert of ~23 kb was isolated. Subsequent subcloning enabled isolation of a 2.5 kb *Pst* I genomic rice fragment showing strong cross-hybridization with the barley *Mlo* gene probe. DNA sequencing of this fragment revealed remarkable DNA sequence similarities within exon sequences of the barley *Mlo* gene (Figure 5).

Finally, a 13 kb λ genomic barley clone derived from cultivar Igri (Stratagene) was isolated with a labelled barley genomic fragment containing *Mlo*. The nucleotide sequence derived from a subcloned 2.6 kb Sac I fragment reveals again extensive sequence similarities to the *Mlo* gene (Fig. 6). The

location of the barley *Mlo* homologue within the genome is not within BAC F15 DNA.

In summary, there is conclusive evidence for *Mlo* homologues both in a monocotyledonous and a dicotyledonous plant species.

Discussion

Any speculation as to mode of action of *Mlo* and *mlo* nucleic acid and polypeptides should provide no limitation on the nature or scope of any aspect or embodiment of the present invention.

In plants, resistance to pathogens is frequently determined by dominant resistance genes, whose products are assumed to recognize pathogen-derived avirulence gene products. This mode of pathogen defence follows Flor's gene-for-gene hypothesis (Flor, 1971). Recently, several 'gene-for-gene' type resistance genes have been molecularly isolated (Martin *et al.*, 1993; Bent *et al.*, 1994; Jones *et al.*, 1994; Mindrinos *et al.*, 1994; Whitham *et al.*, 1994; Grant *et al.*, 1995; Lawrence *et al.*, 1995; Song *et al.*, 1995). The surprising finding is that the deduced proteins share remarkable similar structural domains although they trigger resistance reactions to pathogens such as viruses, fungi, and bacteria (Dangl, 1995; Staskawicz *et al.*, 1995). The isolated genes code for proteins that either contain a leucine-rich region (LRR), with or without an attached nucleotide binding site (NBS), indicative of ligand-binding and protein-protein interaction or encode a simple

serine/threonine kinase. A structural combination of LRR and the kinase domain has been reported in the deduced protein from the rice *Xa21* resistance gene (Song *et al.*, 1995). The structural similarity of resistance genes in 'gene-for-gene' defence makes the existence of a common underlying resistance mechanisms likely.

Resistance mediated by recessive resistance alleles of the *Mlo* gene differs in various aspects from 'gene-for-gene' resistance (see introductory comments above). The molecular isolation of the *Mlo* gene and the sequencing of various mutation-induced *mlo* alleles described here, confirms previous interpretations from combined mutational and Mendelian genetic studies (Hentrich, 1979; Jørgensen, 1983). It is concluded that defective alleles of the *Mlo* locus mediate broad spectrum resistance to pathogens such as the powdery mildew pathogen. This is inconsistent with the involvement of a specific recognition event of a pathogen-derived product as has been proposed for race-specific resistance genes.

Pleiotropic effects of *mlo* alleles have provided some clues towards the development of a molecular concept of the observed broad spectrum resistance response.

Firstly, aseptically grown *mlo* plants exhibit at a high frequency a spontaneous formation of cell wall appositions (CWAs) in leaf epidermal cells (Wolter *et al.*, 1993). Those CWAs are usually formed in response to attempted pathogen penetration directly beneath the fungal apressorium. CWAs are believed to form a physical barrier against pathogen ingress

and have been implicated repeatedly in *mlo* mediated resistance (Bayles, 1990).

Secondly, at a later stage, the plants develop macroscopically detectable leaf necrotic flecks. The spontaneous leaf necrosis response has been extensively studied with a unique collection of 95 chemically-induced *mlo* alleles (Hentrich, 1979). The alleles were classified as either showing a gradually different infection phenotype upon infection of a mixture of nine powdery mildew isolates. Those *mlo* alleles which give rise to an intermediate infection phenotype (i.e. development of a considerable number of sporulating fungal colonies upon inoculation) showed no detectable spontaneous leaf necrosis whereas the category of the most effective resistance alleles exhibits pronounced necrosis in the absence of the pathogen. Thus, there is solid evidence that the former category of *mlo* alleles retain residual wild type allele activity and those alleles appear to exhibit no detectable spontaneous leaf necrosis.

Thirdly, a constitutive expression of defence-related genes has been observed in *mlo* seedlings grown under mildew-free conditions - in primary leaves when 10-11 days old; this includes genes of the *PR-1* family, chitinases and peroxidases.

We have shown that *mlo* in barley confers increased resistance to different types of yellow rust (*Puccinia struciformis*) when a one to one mixture of talcum powder and spores were aviblown onto leaves of *mlo* barley plants after onset of constitutive expression of defence related genes (10-

11 day old *mlo* seedlings).

Thus, it appears that multiple defence-associated responses are constitutively expressed in *mlo* plants.

The temporal relationship of these events is interesting: the onset of constitutive defence-related transcript accumulation is detected in 11 day-old seedlings and precedes CWA formation which is followed by the appearance of macroscopically visible leaf necrosis. Importantly, however, *mlo* resistance can be experimentally tested as early as in five day-old seedlings and is fully functional at this time. We conclude that the Mlo protein has a negative regulatory function in plant defence and that plants with a defective protein are 'primed' for the onset of defence responses.

The deduced amino acid sequence of *Mlo* reveals no significant homologies to any of the described plant resistance genes so far, supporting the idea of a distinct molecular resistance mechanism. The *Mlo* gene shows also no striking similarities to any characterized plant or mammalian gene sequence in the various data bases. However, highly significant homologous sequences have been identified in the EST and genomic databases both from rice and *Arabidopsis thaliana* (Table 5 and Figure 5). This strongly suggests that the Mlo protein represents a member of a novel protein family. A putative nuclear localization motif (NLS) is found within exon 12 providing indication of nuclear localization of the protein (KEKKKVR; Nigg *et al.*, 1991). The significance of this motif is supported by a casein kinase II motif located 14 amino acids

into direction of the NH_2-terminus (SIFD; Rihs *et al.*, 1991). Functional tests may examine the putative subcellular localization of the Mlo protein.

Mutations have been described also in other plant species in which defence responses to pathogens appear to be constitutively expressed (Walbot *et al.*, 1983; Pryor, 1987; Jones, 1994). It has been suggested that this class of mutants, termed lesion mimics (*Les*) or necrotic mutants (*nec*), affect the control of plant defence responses. Recessively inherited lesion mimic mutants have been systematically analysed in *Arabidopsis thaliana* (Greenberg and Ausubel, 1993; Dietrich *et al.*, 1994; Greenberg *et al.*, 1994; Weymann *et al.* 1995). The affected genes have been designated *acd* (accelerated cell death; *acd1* and *acd2*) or *lsd* (lesions simulating disease resistance response; *lsd1* to *lsd7*).

Each of the mutants exhibits, in the absence of pathogens, HR characteristics such as plant cell wall modifications and the accumulation of defence-related gene transcripts. Leaves of the *acd2* mutant have been shown to accumulate high levels of salicylic acid and of the *Arabidopsis* phytoalexin, camelexin (Tsuji *et al.*, 1992). Importantly, *acd* and *lsd* mutants exhibit elevated resistance to a bacterial (*P. syringae*) and fungal (*P. parasitica*) pathogen. The *lsd1* mutant is exceptional in that it confers heightened pathogen resistance at a prelesion state, in contrast to the other defective loci which exhibit elevated pathogen resistance only in the lesion-positive state. In this respect, *lsd1* resembles the *mlo* mutants in barley. Another

striking feature of *lsd1* is the indeterminate spread of lesions in contrast to the other mutants where lesion growth is determinate.

EXPERIMENTAL PROCEDURES

Plant Material

A compilation of the *mlo* mutants and their mother varieties analyzed in this study has been described by Jørgensen (1992) [*mlo*-1, *mlo*-3, *mlo*-4, *mlo*-5, *mlo*-7, *mlo*-8, *mlo*-9, *mlo*-10, *mlo*-11] and by Habekuss and Hentrich (1988) [mutants in cultivar Plena 2018 (*mlo*-13), 2034 (*mlo*-17), 2118]. Since mutant 2118 has not been assigned to an allele number so far, we designate the allele here as *mlo*-26, according to current numbering in the GrainGene database (gopher://greengenes.cit.cornell.edu:70/77/.graingenes.ndx/index?mlo).

The high resolution map is based on a cross between Ingrid *Mlo* x BC_7 Ingrid *mlo*-3. F_1 plants were selfed generating a segregating F_2 population of approximately 600 plants. Phenotypically susceptible F_2 plants which showed heterozygosity for RFLP markers on opposite sites of *Mlo* were selfed and generated further segregants in the F_3 generation for high resolution mapping.

Powdery Mildew Infection Tests

The fungal isolate K1 (Hinze et al., 1991) is virulent on

all cultivars used in this study carrying the *Mlo* allele and avirulent on all tested *mlo* genotypes. Plant growth and inoculation with *Erysiphe graminis* f sp *hordei* were carried out as described previously (Freialdenhoven et al., 1996). The genotype at *Mlo* of recombinants used for the high resolution map were determined after selfing and subsequent inoculation experiments in F_3 or F_4 families comprising at least 24 individuals.

AFLP Analysis

Genomic DNA for AFLP analysis was isolated according to Stewart and Via (1993). AFLP analysis was carried out with minor modifications as described by Vos et al. (1995). For screening of AFLP markers linked to *Mlo* we used the enzyme combinations Pst I/Mse I with amplification primers carrying +2 and +3 selective bases respectively in genomic sequences of amplified fragments. For Eco RI/Mse I amplification primers we used +3 and +3 selective bases respectively. A set of four DNA templates has been used: from the susceptible parent cultivar Ingrid *Mlo*, the resistant parent BC_7Ingrid *mlo*-3, a pool of two resistant F_2 individuals (*mlo*-3 *mlo*-3) and a pool of nine susceptible F_2 individuals (*Mlo Mlo*) derived from the cross Ingrid *Mlo* x BC_7 Ingrid *mlo*-3. Amplified genomic fragments representing AFLP markers Bpm2, Bpm9, and Bpm16 (Figure 1) were cloned and sequenced as follows: gel pieces (fixed by vacuum drying to Whatman 3MM paper) containing the amplified genomic fragments were identified via autoradiography and subsequently

excised. 100 µl water were added, boiled for 10 min. and after centrifugation 5 µl of the supernatant were used as a template for non-radioactive reamplification (30 cycles) with the selective AFLP primers. Amplification products were isolated after agarose gel using a DNA isolation kit (Jetsorb, Genomed Inc., USA). DNA was reated with Klenow polymerase and T4 polynucleotide kinase and subsequently cloned in the EcoRV site of pBluescript SK (Stratagene). Sequencing reactions were performed using a dye terminator cycle sequencing reaction kit (Perkin Elmer) and resolved either on an ABI 373 or 377 (Applied Biosystems) automated sequencer.

Barley YAC Library and BAC Sublibrary Construction of YAC YHV303-A6

The YAC library of barley cultivar Ingrid was established using the pYAC4 vector (Burke et al., 1987; Kuhn and Ludwig 1994) and yeast strain AB 1380. Details of the library construction and its characterization will be described elsewhere. Screening for YAC clones containing marker Bpm16 was done by AFLP analysis. For construction of a BAC sublibrary of YAC YHV303-A6, total DNA of this yeast clone was used. After partial Eco RI digestion and preparative pulsed-field gel electrophoresis, DNA fragments in the size range of 50 kb were recovered and subcloned in the pECSBAC4 vector. Clones carrying YHV303-A6 derived inserts were identified by a two-step colony hybridization procedure. First total labelled DNA of the non-recombinant yeast strain AB 1380 was used as a

probe to eliminate most of the clones carrying insert DNA derived from the host strain. In a subsequent hybridization step the remaining clones were probed with the labelled recombinant chromosome YHV303-A6 after enrichment by preparative pulsed-field gel electrophoresis.

DNA Sequencing of BAC F15

DNA of BAC F15 was isolated by an alkaline lysis large scale plasmid preparation according to Sambrook et al. (1989). 50 µg of purified DNA were nebulized by high pressure treatment with argon gas in a reaction chamber for 150 seconds. The ends of the sheared and reprecipitated DNA were blunt-ended by a T4 DNA polymerase-mediated fill in reaction. DNA fragments in the size range between 800 bp and 3 kb were isolated from agarose gels using a DNA isolation kit (Jetsorb, Genomed Inc., U.S.A.), subcloned into the pBluescript SK vector (Stratagene) and propagated in *E. coli* DN5α. Clones carrying BAC F15 derived inserts were selected by hybridization using the sheared DNA of BAC F15 as a probe. Sequencing reactions were performed as described above. Evaluation of the sequencing data, construction of sequence contigs, and estimation of coding propabilities were done by means of the STADEN software package for Unix users (4th edition, 1994). Assessment of coding probabilities was based on a combined evaluation of uneven positional base frequencies, positional base preference and barley codon usage in the investigated contigs. Homology searches were done using the BLAST software.

PCR-based Sequencing of Alleles at Mlo

Plant chromosomal DNA for this purpose was isolated according to Chunwongse et al. (1993). DNA sequences of *Mlo* alleles of the different barley varieties, *mlo* mutants, BC lines, and intragenic recombinants used in this study were obtained by PCR-based sequencing. Seven overlapping subfragments of the gene (each 400 bp-600 bp in length) were amplified by PCR (35 cycles, 60°C annealing temperature) using sets of specific primers. After preparative agarose gel electrophoresis and isolation of the amplification products using the Jetsorb kit (Genomed Inc., U.S.A.) fragments were reamplified to increase specificity. The resulting products were subsequently purified from nucleotides and oligonucleotides (Jetpure, Genomed Inc., U.S.A.) and used as a template in DNA sequencing reactions (see above). All DNA sequences of mutant alleles and corresponding regions of the parental lines and the intragenic recombinants were derived from both strands and confirmed two times in independent sets of experiments. In addition, mutant alleles *mlo*-1, *mlo*-3, *mlo*-4, *mlo*-5, *mlo*-7, *mlo*-8, *mlo*-9, and *mlo*-10 were also verified in the corresponding BC lines in cultivar Ingrid.

RT-PCR and Rapid Amplification of cDNA Ends (RACE)

RT-PCR was performed using the SUPERSCRIPT preamplification system for first strand cDNA synthesis (Gibco BRL). Total RNA (1 µg) of seven-day-old primary barley leaves (cultivar Ingrid) served as template. First strand cDNA

synthesis was primed by an oligo(dT) primer. The putative coding region of the *Mlo* gene was subsequently amplified using oligonucleotides 25L (GTGCATCTGCGTGTGCGTA) and 38 (CAGAAACTTGTCTCATCCCTG) in a single amplification step (35 cycles, 60°C annealing temperature). The resulting product was analyzed by direct sequencing. 5'- and 3'-ends of the *Mlo* cDNA were determined by RACE (Frohman et al., 1988) using the MARATHON cDNA amplification kit (Clontech). Corresponding experimental procedures were mainly carried out according to the instructions of the manufacturer. To obtain specific RACE products, two consecutive rounds of amplification (35 cycles, 55°C annealing temperature) were necessary. For this purpose, two sets of nested primers were used in combination with the adapter primers of the kit: oligonucleotides 46 (AGGGTCAGGATCGCCAC) and 55 (TTGTGGAGGCCGTGTTCC) for the 5'-end and primers 33 (TGCAGCTATATGACCTTCCCCCTC) and 37 (GGACATGCTGATGGCTCAGA) for the 3'-end. RACE products were subcloned into pBluescript SK (Stratagene). Ten 5'-end and eight 3' end clones were chosen for DNA sequence analysis.

The term "AFLPs" is used herein to refer to "AFLP markers".

Table 1 summarizes the identified mutation sites of various mutants within the *Mlo* gene. The origin, the mutagen and the predicted effect of the mutation at the amino acid level are indicated.

Table 2 shows the results of heteroallelic *mlo* crosses and selfings of the respective *mlo* lines to isolate intragenic recombination events.

Table 3 summarizes the genotypes at flanking RFLP markers in susceptible F_2 or homozygous F_3 progeny from the intermutant crosses. CO and NCO indicate crossover type and non crossover type recombinants deduced from flanking molecular marker exchange. Table 3 summarizes DNA sequence analysis of suceptible intragenic crossover type recombinants (from homozygous susceptible F_3 progeny) and the corresponding parental *mlo* mutant lines. Sequences flanking the identified mutation sites are shown.

Table 4 shows the results of direct PCR sequencing of genomic DNA of susceptible intragenic recombinants derived from both heteroallelic crosses *mlo-1 x mlo-8* and *mlo-1 x mlo-5*, revealing restoration of wild type sequences.

Table 5 shows several *Arabidopsis thaliana* and two rice expressed sequence tags (ESTs) with homology to the Mlo protein.

Table 5A show amino acid sequences, with "query" indicating part of the Mlo protein sequence to which homology has been found, with the predicted amino acid sequence of each identified EST marked with "subject".

Table 5B shows EST nucleotide sequences encoding the amino acid sequences shown in Table 5A. GenBank Accession number T22145 (definition 4153 Arabidopsis thaliana cDNA clone 97N8T7, NCBI Seq ID 932185), number T22146 (definition 4153 Arabidopsis thaliana cDNA clone 97N9T7, NCBI Seq ID 932186), number N37544 (definition 18771 Arabidopsis thaliana cDNA clone 205N12T7, NCBI Seq ID 1158686), number T88073 (definition 11769 Arabidopsis thaliana cDNA clone 155I23T7, NCBI Seq ID 935932) number H76041 (definition 17746 Arabidopsis thaliana cDNA clone 193P6T7, NCBI seq ID 1053292), number D24287 (rice cDNA partial sequence R1638_1A, nID g428139) and D24131 (rice cDNA partial sequence R1408_1A, nID g427985) are shown. The Arabidopsis sequences are from Newman *et al.* (1994) *Plant Physiol.* 106 1241-55. The rice sequences are from Minobe, Y. and Sasaki, T. submitted 2 Nov 1993 to DDBJ.

Table 1. *mlo* Mutant Alleles

Allele	Mother Variety	Mutagen	Mutational Event at *Mlo*	Effect on Amino Acid Level
mlo-1	Haisa	X-rays	$T^{484} \rightarrow A$	$trp^{162} \rightarrow arg$
mlo-3	Malteria Heda	γ-rays	deletion of 2 nucleotides (1188-1189)	frame shift after phe^{395}
mlo-4	Foma	X-rays	deletion of 11 nucleotides (478-488)	frame shift after trp^{159}
mlo-5	Carlsberg II	EMS	$G^{3} \rightarrow A$	$met^{1} \rightarrow ile^{a}$
mlo-7	Carlsberg II	EMS	$G^{677} \rightarrow A$	$gly^{226} \rightarrow asp$
mlo-8	Carlsberg II	EMS	$A^{1} \rightarrow G$	$met^{1} \rightarrow val^{a}$
mlo-9	Diamant	EMS	$C^{28} \rightarrow T$	$arg^{10} \rightarrow trp$
mlo-10	Foma	γ-rays	deletion of 6 nucleotides (543-548)	2 amino acids (phe^{182}, thr^{183}) missing
mlo-12	Elgina	NMU	$C^{720} \rightarrow A$	$phe^{240} \rightarrow leu$
mlo-13	Plena	EMS	$T^{89} \rightarrow A$	$val^{30} \rightarrow glu$
mlo-16	Alsa	EMS	$G^{1917*} \rightarrow A$	alteration in 3' splice border of intron 9
mlo-17	Plena	EMS	$C^{92} \rightarrow T$	$ser^{31} \rightarrow phe$
mlo-26	Plena	EMS	$T^{809} \rightarrow A$	$leu^{270} \rightarrow his$
mlo-28	Nadja	NaN_3	$C^{665} \rightarrow T$	$thr^{222} \rightarrow ile$

Numbers of nucleotides and amino acids are given according to the translational start site of the *Mlo* cDNA sequence.

* Nucleotide number according to the translational start site of the genomic *Mlo* DNA sequence.

EMS = ethylmethane sulfonate, NMU = nitrosomethylurea, NaN_3 = sodium azide.

[a] Next start codon is at nucleotide positions 79-81 and is in frame with the coding sequence.

Table 2

F_2 progeny from intermutant crosses and selfings

Testcrosses	resistant	*susceptible*	frequency of susceptible F_2 progeny
mlo-8 x *mlo-1*	5,281	3	$5,7 \times 10^{-4}$
mlo-5 x *mlo-1*	915	0	---
mlo-5 x mlo-1	14,474	9	$6,2 \times 10^{-4}$

selfings	resistant	susceptible
mlo-1	12,634	0
mlo-5	5,498	0
mlo-8	8,435	0

Table 3. Genotypes at Flanking RFLP Markers in Susceptible Progeny Derived from Heteroallelic *mlo* Crosses

Testcross	Susceptible Plant	Parental Genotype in Centromeric Orientation to *Mlo*[1]	Parental Genotype in Telomeric Orientation to *Mlo*[2]	Type of Recombination
mlo-8 x *mlo-1*	1	*mlo-1*	*mlo-8*	CO
	2	*mlo-1*	*mlo-8*	CO
	3	*mlo-8*	*mlo-8*	NCO
mlo-1 x *mlo-5*	1	*mlo-1*	*mlo-5*	CO
	2	*mlo-1*	*mlo-5*	CO
	3	*mlo-5*	*mlo-5*	NCO
	4	*mlo-1*	*mlo-5*	CO
	5	*mlo-5*	*mlo-5*	NCO
	6	*mlo-5*	*mlo-5*	NCO
	7	*mlo-1*	*mlo-5*	CO
	8*	*mlo-5*	*mlo-5*	NCO
	9*	*mlo-1* + *mlo-5*	*mlo-5*	CO

[1]deduced from alleles of RFLP marker WG114 (see Figure 1)

[2]deduced from alleles of RFLP marker ABG366 (see Figure 1)

CO = cross over type, NCO = non cross over type of recombination

*Genotypes of flanking RFLP markers have been determined in heterozygous susceptible F_2 individuals; in all other cases homozygous susceptible F_3 progeny derived from the susceptible F_2 individuals were tested

Table 4. Restoration of *Mlo* Wild Type Sequences by Intragenic Recombinantion Events

			Nucleotide Sequences Flanking Mutant Sites[1]	
	Genotypes		Nucleotides -3 to +3	Nucleotides 481 to 486
Haisa	*Mlo*		CCGATG	AATGGG
	mlo-1		CCGATG	AAAGGG
Carlsberg II	*Mlo*		CCGATG	AATGGG
	mlo-5		CCGATA	AATGGG
	mlo-8		CCGGTG	AATGGG
Intragenic recombinant	*mlo-1* x *mlo-8*	1	CCGATG	AATGGG
		2	CCGATG	AATGGG
	mlo-1 x *mlo-5*	1	CCGATG	AATGGG
		2	CCGATG	AATGGG
		4	CCGATG	AATGGG
		7	CCGATG	AATGGG
		9	CCGATG	AATGGG

[1]Numbers of nucleotides are given according to the translational start site (see Figure 2)

TABLE 5A

```
>EM EST1:AT1452 T22145 4153 Arabidopsis thaliana cDNA clone 97N8T7. 11/95
  Length = 382

  Plus Strand HSPs:

 Score = 248 (115.9 bits), Expect = 2.9e-27, P = 2.9e-27
 Identities = 47/100 (47%), Positives = 67/100 (67%), Frame = +2

Query:   242 KYIKRSMEDDFKVVVGISLPLWGVAILTLFLDINGVGTLIWISFIPLVILLCVGTKLEMI 301
             KY+ R++EDDFK VVGIS  LW   ++   L++NG  T  WI+FIP  +LL VGTKLE +
Sbjct:     2 KYMMRALEDDFKQVVGISWYLWXFVVIFXLLNVNGWHTYFWIAFIPFXLLLAVGTKLEHV 181

Query:   302 IMEMALEIQDRASVIKGAPVVEPSNKFFWFHRPDWVLFFI 341
             I ++A E+ ++   I+G  VV+P . + FWF +P  VL+ I
Sbjct:   182 IAQLAHEVAEKHVAIEGDLVVKPXXEHFWFSKPQIVLYLI 301


>EM EST1:AT1462 T22146 4154 Arabidopsis thaliana cDNA clone 97N9T7. 11/95
  Length = 390

  Plus Strand HSPs:

 Score = 212 (99.1 bits), Expect = 4.2e-26, Sum P(2) = 4.2e-26
 Identities = 41/83 (49%), Positives = 58/83 (69%), Frame = +2

Query:   242 KYIKRSMEDDFKVVVGISLPLWGVAILTLFLDINGVGTLIWISFIPLVILLCVGTKLEMI 301
             KY+ R++EDDFK VVGIS  LW   ++ L L++NG  T  WI+FIP  +LL VGTKLE +
Sbjct:     2 KYMMRALEDDFKQVVGISWYLWXFVVIFLLLNVNGWHTYFWIAFIPFALLLAVGTKLEHV 181

Query:   302 IMEMALEIQDRASVIKGAPVVEP 324
             I ++A E+ ++   I+G  VV+P
Sbjct:   182 IAQLAHEVAEKHVAIEGDLVVKP 250

 Score = 52 (24.3 bits), Expect = 1.9, Sum P(2) = 0.85
 Identities = 9/32 (28%), Positives = 16/32 (50%), Frame = +2

Query:    18 WAVAVVFAAMVLVSVLMEHGLHKLGHWFQHRH 49
             W   + FA ++ V   +EH + +L H    +H
Sbjct:   122 WIAFIPFALLLAVGTKLEHVIAQLAHEVAEKH 217


 Score = 49 (22.9 bits), Expect = 4.2e-26, Sum P(2) = 4.2e-26
 Identities = 8/17 (47%), Positives = 12/17 (70%), Frame = +1

Query:   323 EPSNKFFWFHRPDWVLF 339
             E S++ EWF +P  VL+
Sbjct:   244 ETSDEHFWFSKPQXVLY 294
```

TABLE 5A cont'd

```
>EM EST1:AT54418 N37544 18771 Arabidopsis thaliana cDNA clone 205N12T7. 1/96
  Length = 585

  Plus Strand HSPs:

 Score = 277 (129.5 bits), Expect = 1.2e-45, Sum P(2) = 1.2e-45
 Identities = 51/96 (53%), Positives = 71/96 (73%), Frame = +1

Query:   236 SKFDFHKYIKRSMEDDFKVVVGISLPLWGVAILTLFLDINGVGTLIWISFIPLVILLCVG 295
             S+FDF KYI+RS+E DFK VV IS  +W VA+L L  +  G+ + +W+ FIPLV++L VG
Sbjct:   127 SRFDFRKYIQRSLEKDFKTVVEISPVIWFVAVLFLLTNSYGLRSYLWLPFIPLVVILIVG 306

Query:   296 TKLEMIIMEMALEIQDRASVIKGAPVVEPSNKFFWF 331
             TKLE+II ++ L IQ+   V++GAPVV+P +  FWF
Sbjct:   307 TKLEVIITKLGLRIQEEGDVVRGAPVVQPGDDXFWF 414

 Score = 121 (56.6 bits), Expect = 1.2e-45, Sum P(2) = 1.2e-45
 Identities = 25/45 (55%), Positives = 29/45 (64%), Frame = +1

Query:   196 SSTPGIRWVVAFFRQFFRSVTKVDYLTLRAGFINAHLSQNSKFDF 240
             S T    W+V FFRQFF SVTKVDYL L  GFI AH +  ++  F
Sbjct:     1 SKTRVTLWIVCFFRQFFGSVTKVDYLALXHGFIMAHFAPGNESRF 135


>EM EST1:AT04117 H76041 17746 Arabidopsis thaliana cDNA clone 193P6T7. 11/95
  Length = 476

  Plus Strand HSPs:
 Score = 210 (98.2 bits), Expect = 9.0e-36, Sum P(2) = 9.0e-36
 Identities = 43/86 (50%), Positives = 58/86 (67%), Frame = +1

Query:   196 SSTPGIRWVVAFFRQFFRSVTKVDYLTLRAGFINAHLSQNSKFDFHKYIKRSMEDDFKVV 255
             ++TP    V  FFRQFF SV + DYLTLR GF +AHL+   KF+F +YIK S+EDDFK+V
Sbjct:   124 TTTPFXFNVGCFFRQFFVSVERTDYLTLRHGFXSAHLAPGRKFNFQRYIKXSLEDDFKLV 303

Query:   256 VGISLPLWGVAILTLFLDINGVGTLI 281
             VGI   LW   ++ L +    +GT++
Sbjct:   304 VGIXPVLWASFVIFLAVQX*WLGTIV 381

 Score = 119 (55.6 bits), Expect = 9.0e-36, Sum P(2) = 9.0e-36
 Identities = 24/57 (42%), Positives = 32/57 (56%), Frame = +1

Query:   156 MRTWKKWETETTSLEYQFANDPARFRFTHQTSFVKRHLGLSSTPGIRWVVAFFRQFF 212
             +R WKKWE   T S +Y F   D +R R TH+TSFV+ H     +T    + V  F + F
Sbjct:     1 IRGWKKWEQXTLSNDYXFXIDHSRLRLTHETSFVREHTSFWTTTPFXFNVGCFFRQF 171


 Score = 40 (18.7 bits), Expect = 1.2e-08, Sum P(2) = 1.2e-08
 Identities = 8/19 (42%), Positives = 10/19 (52%), Frame = +2
Query:   269 TLFLDINGVGTLIWISFIP 287
             +L  + NG G L W S  P
Sbjct:   344 SLLFNXNGWGPLFWASVPP 400
```

TABLE 5A cont'd

```
>EM EST1:AT0739 T88073 11769 Arabidopsis thaliana cDNA clone 155I23T7. 11/95
  Length = 460

  Plus Strand HSPs:

 Score = 175 (81.8 bits), Expect = 1.2e-24, Sum P(2) = 1.2e-24
 Identities = 31/67 (46%), Positives = 43/67 (64%), Frame = +1

Query:   146 VITIALSRLKMRTWKKWETETTSLEYQFANDPARFRFTHQTSFVKRHLGLSSTPGIRWVV 205
             ++T A  ++KMRTWK WE ET ++EYQ++NDP RFRF   TSF +RHL   S   +   +
Sbjct:     4 IVTYAFGKIKMRTWKSWEEETKTIEYQYSNDPERFRFARDTSFGRRHLNFWSKTRVTLWI 183


 Score = 121 (56.6 bits), Expect = 1.4e-14, Sum P(2) = 1.4e-14
 Identities = 25/45 (55%), Positives = 29/45 (64%), Frame = +1

Query:   196 SSTPGIRWVVAFFRQFFRSVTKVDYLTLRAGFINAHLSQNSKFDF 240
             S T    W+V FFRQFF SVTKVDYL L  GFI AH +  ++  F
Sbjct:   157 SKTRVTLWIVCFFRQFFGSVTKVDYLALXHGFIMAHFAPGNESRF 291

 Score = 75 (35.1 bits), Expect = 1.2e-24, Sum P(2) = 1.2e-24
 Identities = 14/21 (66%), Positives = 17/21 (80%), Frame = +1

Query:   236 SKFDFHKYIKRSMEDDFKVVV 256
             S+FDF KYI+RS+  DFK VV
Sbjct:   283 SRFDFRKYIQRSLXXDFKTVV 345


>EM EST5:OSR16381A D24287 Rice cDNA, partial sequence (R1638_1A). 5/95
  Length = 400
  Plus Strand HSPs:

 Score = 147 (68.7 bits), Expect = 1.9e-16, Sum P(2) = 1.9e-16
 Identities = 26/53 (49%), Positives = 35/53 (66%), Frame = +1

Query:   236 SKFDFHKYIKRSMEDDFKVVVGISLPLWGVAILTLFLDINGVGTLIWISFIPL 288
             ++F+F KYIKR +EDDFK VVGIS P W  A+  +  +++G   L W S  PL
Sbjct:   202 TRFNFRKYIKRXLEDDFKTVVGISAPXWASALAIMLFNVHGWHNLFWFSTXPL 360

 Score = 45 (21.0 bits), Expect = 1.9e-16, Sum P(2) = 1.9e-16
 Identities = 9/15 (60%), Positives = 11/15 (73%), Frame = +2

Query:   287 PLVILLCVGTKLEMI 301
             PL + L VGTKL+ I
Sbjct:   356 PLXVTLAVGTKLQAI 400


>EM EST5:OSS1692A D39989 Rice cDNA, partial sequence (S1692_1A). 11/94
  Length = 343

  Plus Strand HSPs:
 Score = 95 (44.4 bits), Expect = 0.00059, P = 0.00059
 Identities = 24/58 (41%), Positives = 31/58 (53%), Frame = +3

Query:    43 HWFQHRHKKALWEALEKMKAELMLVGFISLLLIVTQDPIIAKICISEDAADVMWPCKR 100
             H  +  H+  L +A+EKMK E+ML+GFISLLL  T   I       S+        PC R
Sbjct:     3 HXSEKTHRNPLHKAMEKMKEEMMLLGFISLLLAATSRIISGICIDSKYYNSNFSPCTR 176
```

TABLE 5B

GenBank Accession Number T22145

```
  1 caagtatatg atgcgcgctc tagaggatga tttcaaacaa gttgttggta ttagttggta
 61 tctttggntc tttgtcgtca tctttttnct gctaaatgtt aacggatggc acacatattt
121 ctggatagca tttattccct ttnctttgct tcttgctgtg ggaacaaagt tggagcatgt
181 nattgcacag ttagctcatg aagttgcaga gaaacatgta gccattgaag gagacttagt
241 ggtgaaaccc ncanatgagc atttctggtt cagcaaacct caaattgttc tctacttgat
301 cccatttttat cctctttccc agaatgcntt ttnagantgc nttttttnnt tttggnnttt
361 ggggtaanan annggtttcg nc
```

GenBank Accession Number T22146

```
  1 caagtatatg atgcgcgctc tagaggatga tttcaaacaa gttgttggta ttagttggta
 61 tctttggntc tttgtcgtca tctttttgct gctaaatgtt aacggatggc acacatattt
121 ctggatagca tttattccct ttgctttgct tcttgctgtg ggaacaaagt tggagcatgt
181 nattgcacag ttagctcatg aagttgcaga gaaacatgta gccattgaag gagacttagt
241 ggtgaaacct cagatgagca tttctggttc agcaaacctc aaantgttct ctactngatc
301 cnctttatcc cccttccaga atgccttttt nangattcnn nttttttctt nttgganntt
361 ttgggnnnnc aaacgggntt nggacctccg
```

GenBank Accession Number N37544

```
  1 agcaagacga gagtcacact atggattgtt tgtttttta gacagttctt tggatctgtc
 61 accaaagttg attacttagc actaagncat ggtttcatca tggcgcattt tgctcccggt
121 aacgaatcaa gattcgattt ccgcaagtat attcagagat cattagagaa agacttcaaa
181 accgttgttg aaatcagtcc ggttatctgg tttgtcgctg tgctattcct cttgaccaat
241 tcatatggat tacgttctta cctctggtta ccattcattc cactagtcgt aattctaata
301 gttggaacaa agcttgaagt cataataaca aaattgggtc taaggatcca agaggaaggt
361 gatgtggtga gaggcgcccc agtggttcag cctggtgatg accncttctg gtttngnaan
421 cacgnttcaa tnttttccnt antcacttng gccttttan gggtgaattt caacttcatn
481 ctttncctgg ggncggatga ttcaatccaa naatnttccc ctgaagnctn caagtttggg
541 cataggcttt nggtgggntt ttcaganttt nagtttggct tnccc
```

TABLE 5B (Continued)

GenBank Accession Number T88073

```
  1 tgcattgtta cttatgcttt cggaaagatc aagatgagga cgtggaagtc gtgggaggaa
 61 gagacaaaga caatagagta tcagtattcc aacgatcctg agaggttcag gtttgcnagg
121 gacacatctt ttgggagaag acatctcaat ttctggagca agacgagagt cacactatgg
181 attgtttgtt tttttagaca gttctttgga tctgtcacca aagttgatta cttagcacta
241 agncatggtt tcatcatggc gcattttgct cccggtaacg aatcaagatt cgatttccgc
301 aagtatattc agagatcatt agngnaagac ttcaaaaccg ttgtttgaaa tcagtccggt
361 tatctggttt gtcggctgtg ctattccnct tgaccaattc atatggntnc ggtnttncnc
421 tggtaccatt attcnctagc ggaatntaaa agttggcnga
```

GenBank Accession Number H76041

```
  1 attcgtggat ggaaaaagtg ggagcaagan acattatcta atgactatna gtttnctatt
 61 gatcattcaa gacttaggct cactcatgag acttcttttg tnagagaaca tacaagtttc
121 tggacaacaa cncctttctn ctttaacgtc ggatgcttct ttaggcagtt ctttgtatct
181 gtngaaagaa ccgactactt gactctgcgc catggattca nctctgccca tttagctcca
241 ggaagaaagt tcaacttcca gagatatatc aaangatctc tcgaggatga tttcaagttg
301 gtagttggaa taagnccagt tctttgggca tcatttgtaa tcttccttgc tgttcaatgn
361 taatggctgg ggaccattgt tttgggcntc ggtaccgcct ntactcanaa ncccaggctt
421 ttggccaagg ttcaaggaat ttngggacaa tggggtagaa tcgtgggcnc atnngg
```

GenBank Accession Number D24287

```
  1 tcntntttnn ttttcgnntn cntccacccc tnnnntnctc nancncnttn nnnttatctc
 61 tnttnttntc ncntntcccn ncaccaccnn ncgacgggcn tggactnngc ccnnngttcg
121 aggctgccca ctgncgtctg agacctacct tgncatttga cggcacngga cttcanttgc
181 tgctcacttt atctctacgg gactaggttc aattttcgga aatacatcaa aaggncactg
241 gaggacgatt ttaagacagt tgttggcatt agtgcacccn tatgggcttc tgcgttggcc
301 attatgctct tcaatgttca tggatggcat aacttgttct ggttctctac aatnccccctt
361 gntagtaact ttagcagttg gaacaaagct gcaggctata
```

GenBank Accession Number D24131

```
  1 cagactacct gactttgagg cacggattca ttgctgctca tttatctcta gggactaggt
 61 tcaattttcg gaaatacatc aaaaggtcac tggaggacga ttttaagaca gttgttggca
121 ttagtgcacc cttatgggct tctgcgttgg ccattatgct cttnaatgtt catggatggc
181 ataacttgtt ctggttctct acaatccccc ttgtagtaac tttagcagtt ggaacaaagc
241 tgcaggctat aattgcaatg atggctgttg aaattaaaga gaggcataca gtaattcaag
301 gaatgccggt ggtgaactca gtgat
```

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